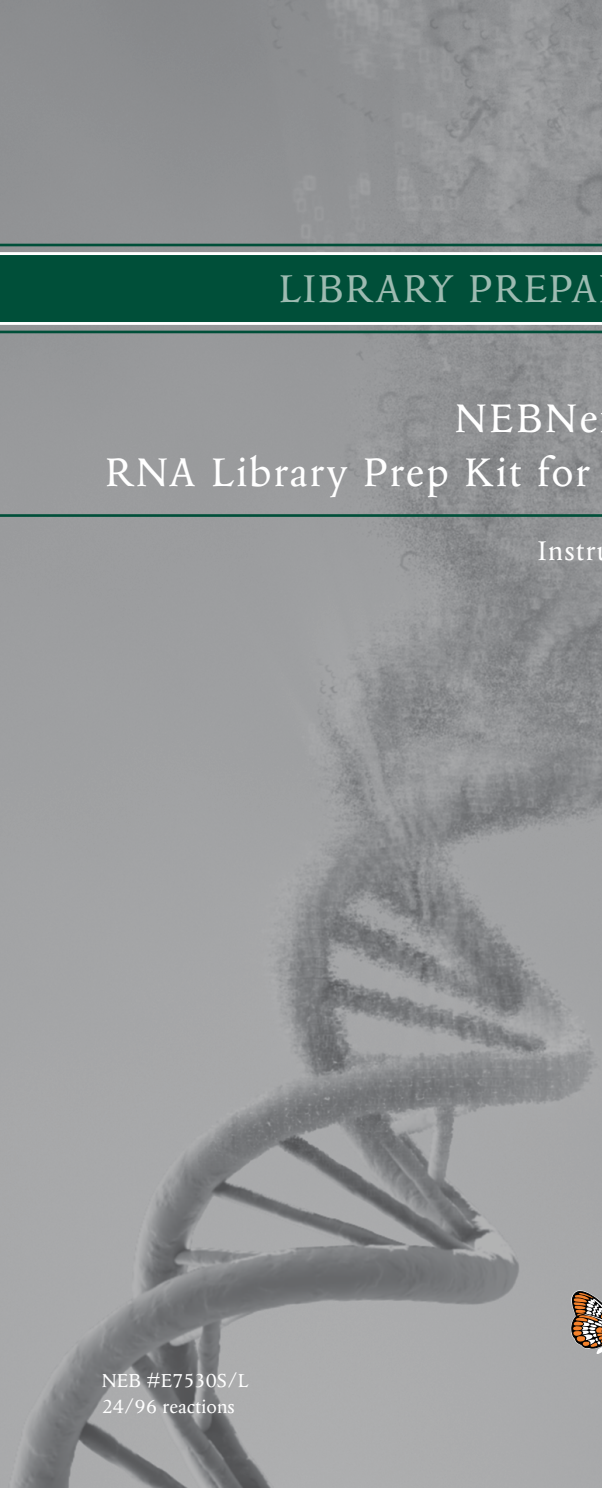
LIBRARY PREPARATION

NEBNext® Ultra™ RNA Library Prep Kit for Illumina®

Instruction Manual



NEB #E7530S/L
24/96 reactions

NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

Sign up for the NEBNext e-newsletter

Scan this code or visit www.neb.com/NEBNextnews2 to sign up for the NEBNext bimonthly e-newsletter to learn about new NEBNext products, recent publications and advances in library prep for next gen sequencing.

NEBNext Ultra RNA Library Prep Kit for Illumina

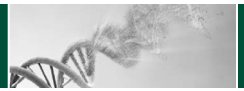


Table of Contents:

The Library Prep Kit Includes:

The volumes provided are sufficient for preparation of up to 24 reactions (NEB #E7530S) and 96 reactions (NEB #E7530L). (All reagents should be stored at –20°C).

- (pink) NEBNext First Strand Synthesis Reaction Buffer (5X)
- (pink) Random Primers
- (pink) ProtoScript II Reverse Transcriptase
- (pink) Murine RNase Inhibitor RRI
- (orange) NEBNext Second Strand Synthesis Enzyme Mix
- (orange) NEBNext Second Strand Synthesis Reaction Buffer
- (green) NEBNext End Prep Enzyme Mix
- (green) NEBNext End Repair Reaction Buffer (10X)
- (red) Blunt/TA Ligase Master Mix

Nuclease-free water

- (blue) NEBNext High-Fidelity 2X PCR Master Mix

Required Materials Not Included:

NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490)

NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500) Oligos for Illumina

Magnetic Rack (Alpaqua, cat #A001322 or equivalent)

80% Ethanol (freshly prepared)

Agencourt® AMPure® XP Beads (Beckman Coulter, Inc. #A63881)

Applications:

The NEBNext Ultra RNA Library Prep Kit for Illumina contains enzymes and buffers that are ideally suited for cDNA library preparation for next-generation sequencing. Each of these components must pass rigorous quality control standards and are lot controlled, both individually and as a set of reagents.

Lot Control: The lots provided in the NEBNext Ultra RNA Library Prep Kit for Illumina are managed separately and are qualified by additional functional validation. Individual reagents undergo standard enzyme activity and quality control assays, and also meet stringent criteria in the additional quality controls listed on each individual component page.

Functionally Validated: Each set of reagents is functionally validated together through construction and sequencing of a transcriptome library on an Illumina sequencing platform.

For larger volume requirements, customized and bulk packaging is available by purchasing through the OEM/Bulks department at NEB. Please contact OEM@ neb.com for further information.

Protocol:

Please refer to revision history for a summary of protocol updates

Symbols

This is a point where you can safely stop the protocol and store the samples prior to proceeding to the next step in the protocol.

This caution sign signifies a step in the protocol that has two paths leading to the same end point but is dependent on a user variable, like the type of RNA input.

Colored bullets indicate the cap color of the reagent to be added

The protocol has been optimized using high quality Universal Human Reference Total RNA. For PolyA mRNA selection, high quality RNA with RIN score > 7 (measured by bioanalyzer) is required.

Starting Material: Total RNA (10 ng–1 µg), purified mRNA (10–100 ng), or ribosomal depleted total RNA (10–100 ng) quantified by bioanalyzer.

The protocol is optimized for approximately 200 bp RNA inserts. To generate libraries with longer RNA insert sizes, refer to Appendix A for recommended fragmentation times and size selection conditions.

Note: Follow steps in Protocol (A) if starting material is total RNA. Perform mRNA isolation, fragmentation and priming using the NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB #E7490). If starting material is purified mRNA or ribosomal depleted RNA, proceed to (B) on page 6.

(A) Preparation of First Strand Reaction Buffer and Random Primer Mix

Prepare the First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) as follows in a nuclease-free tube:

(pink) NEBNext First Strand Synthesis Reaction Buffer (5X)	8 µl
(pink) NEBNext Random Primers	2 µl
Nuclease-free water	10 µl
Total Volume	20 µl

Note: Keep the mix on ice during the mRNA isolation.

mRNA Isolation, Fragmentation and Priming Starting with Total RNA

1. Dilute the total RNA with nuclease-free water to a final volume of 50 µl in a nuclease-free 0.2 ml PCR tube and keep on ice.
2. Aliquot 15 µl of NEBNext Oligo $d(T)_{25}$ beads into a nuclease-free 0.2 ml PCR tube.
3. Wash the beads by adding 100 µl of 2X RNA Binding Buffer to the beads. Pipette the entire volume up and down 6 times to mix thoroughly.
4. Place the tubes on the magnetic rack at room temperature for 2 minutes.
5. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
6. Remove the tube from the magnetic rack.
7. Repeat steps 3–6.
8. Resuspend the beads in 50 µl of 2X RNA binding Buffer and add the 50 µl of total RNA sample from step 1.
9. Place the tube on a thermal cycler and close the lid. Heat the sample at 65°C for 5 minutes and hold at 4°C to denature the RNA and facilitate binding of the poly-A mRNA to the beads.
10. Remove the tube from the thermal cycler when the temperature reaches 4°C.
11. Place the tube on the bench and incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.
12. Place the tube on the magnetic rack at room temperature for 2 minutes to separate the poly-A mRNA bound to the beads from the solution.
13. Remove and discard all of the supernatant. Take care not to disturb the beads.
14. Remove the tube from the magnetic rack.
15. Wash the beads by adding 200 µl of Wash Buffer to the tube to remove unbound RNA. Pipette the entire volume up and down 6 times to mix thoroughly.
16. Place the tube on the magnetic rack at room temperature for 2 minutes.
17. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
18. Remove the tube from the magnetic rack.
19. Repeat steps 15–18.
20. Add 50 µl of elution buffer to each tube. Gently pipette the entire volume up and down 6 times to mix thoroughly.
21. Place the tube on the thermal cycler. Close the lid and heat the samples at 80°C for 2 minutes, then hold at 25°C to elute the Poly-A mRNA from the beads.
22. Remove the tube from the thermal cycler when the temperature reaches 25°C.
23. Add 50 µl of 2X RNA Binding Buffer to the sample to allow the mRNA to re-bind to the beads. Gently pipette the entire volume up and down 6 times to mix thoroughly.
24. Incubate the tube at room temperature for 5 minutes.
25. Place the tube on the magnetic rack at room temperature for 2 minutes.

26. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
27. Remove the tube from the magnetic rack.
28. Wash the beads by adding 200 µl of Wash Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.
29. Place the tube on the magnetic rack at room temperature for 2 minutes.
30 Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.
31. Remove the tubes from the magnetic rack.
32. Wash the beads by adding 200 µl of Elution Buffer. Gently pipette the entire volume up and down 6 times to mix thoroughly.

Note: This is not an elution step. Elution buffer is being used as an additional wash step.

33. Place the tube on the magnetic rack at room temperature for 2 minutes.
34. Remove and discard all of the supernatant from the tube. Take care not to disturb the beads.

Note: It is important to remove all of the supernatant to successfully fragment the mRNA in the subsequent steps. Spin down the tube. Place the tube on the magnetic rack and with a 10 µl tip remove all of the elution buffer. Caution: Do not disturb beads that contain the mRNA.

35. Remove the tube from the magnetic rack.

Note: For RNA insert sizes > 200 nt, refer to Appendix A for recommended fragmentation time.

36. Elute mRNA from the beads by adding 15 µl of the First Strand Synthesis Reaction Buffer and Random Primer mix (2X) prepared at the start of the protocol (page 4) and incubating the sample at 94°C for 15 minutes. Immediately, place the tubes on the magnetic rack.
37. Collect the purified mRNA by transferring 10 µl of the supernatant to a clean nuclease-free PCR Tube.
38. Place the tube on ice.
39. Proceed to First Strand cDNA Synthesis

⚠ (B) RNA Fragmentation and Priming Starting from Purified mRNA or ribosomal depleted mRNA:

Purified mRNA/ribosomal depleted RNA (10–100 ng)	5 µl
● (pink) NEBNext First Strand Synthesis Reaction Buffer (5X)	4 µl
● (pink) Random Primers	1 µl
Final volume	10 µl

Note: Refer to Appendix A for fragmentation conditions if you are preparing libraries with larger inserts (> 200 nt).

⚠ 1. Incubate the sample at 94°C for 15 minutes.
2. Transfer the tube to ice.
3. Proceed to First Strand cDNA Synthesis

First Strand cDNA Synthesis

1. To the fragmented and primed mRNA (10 µl from section A step 38 or section B step 2) add the following components:

● (pink) Murine RNase Inhibitor	0.5 µl
● (pink) ProtoScript II Reverse Transcriptase	1 µl
Nuclease free water	8.5 µl
Final volume	20 µl

Note: If you are following recommendations in Appendix A, for longer RNA fragments, incubate for 50 minutes at 42°C.

2. ⚠ Incubate the sample in a preheated thermal cycler as follows:

   10 minutes at 25°C
   15 minutes at 42°C
   15 minutes at 70°C
   Hold at 4°C

3. Immediately, perform second strand synthesis reaction.

Perform Second Strand cDNA Synthesis

1. Add the following reagents to the First Strand Synthesis reaction (20 µl):

Nuclease-free water	48 µl
● (orange) Second Strand Synthesis Reaction Buffer (10X)	8 µl
● (orange) Second Strand Synthesis Enzyme Mix	4 µl
Total volume	80 µl

2. Mix thoroughly by gentle pipetting.
3. Incubate in a thermal cycler for 1 hour at 16°C, with heated lid set at ≤ 40°C.

Purify the Double-stranded cDNA Using 1.8X Agencourt AMPure XP Beads

1. Vortex AMPure XP beads to resuspend.
2. Add 144 µl (1.8X) of resuspended AMPure XP beads to the second strand synthesis reaction (~80 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge to collect any sample on the sides of the tube. Place the tube on an appropriate magnetic rack to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Air dry the beads for 10 minutes while the tube is on the magnetic rack with lid open.
8. Elute the DNA target from the beads into 60 µl nuclease-free water. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and then place it in the magnetic rack until the solution is clear.
9. Remove 55.5 µl of the supernatant and transfer to a clean nuclease-free PCR tube.

Note: If you need to stop at this point in the protocol samples can be stored at –20°C.

Perform End Repair/dA-tail of cDNA Library

1. To the purified double-stranded cDNA (55.5 µl), add the following components:

(green) NEBNext End Repair Reaction Buffer (10X)	6.5 µl
(green) NEBNext End Prep Enzyme Mix	3 µl
Total volume	65 µl

2. Incubate the sample in a thermal cycler as follows:
   30 minutes at 20°C
   30 minutes at 65°C
   Hold at 4°C
3. Proceed immediately to Adaptor Ligation.

Perform Adaptor Ligation

Dilute the NEBNext Adaptor for Illumina (15 µM) to 1.5 µM with a 10-fold dilution (1:9) with sterile water for immediate use.

1. To the dA-Tailed cDNA (65 µl), add the following components:

(red) Blunt/TA Ligase Master Mix	15 µl
(red) Diluted NEBNext Adaptor*	1 µl
Nuclease-free Water	2.5 µl
Total volume	83.5 µl

*The adaptor is provided in NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500) Oligos for Illumina.

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
3. Incubate 15 minutes at 20°C in a thermal cycler.
4. Add 3 µl of (red) USER Enzyme to the ligation mixture from Step 3.
5. Mix well and incubate at 37°C for 15 minutes.

Purify the Ligation Reaction Using AMPure XP Beads

Note: If you are selecting for larger size fragments (> 200 nt) follow the size selection recommendations in Appendix A.

1. To the ligation reaction (86.5 µl), add 13.5 µl nuclease-free water to bring the reaction volume to 100 µl.

Note: X refers to the original sample volume of 100 µl from the above step.

2. Add 100 µl (1.0X) resuspended AMPure XP beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contain unwanted fragments **(Caution: do not discard the beads)**.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Briefly spin the tube, and put the tube back in the magnetic rack.
8. Completely remove the residual ethanol, and air dry beads for 10 minutes while the tube is on the magnetic rack with the lid open.
9. Elute DNA target from the beads with 50 µl nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.
10. Transfer the 50 µl supernatant to a clean PCR tube. Discard beads.

11. To the 50 µl supernatant, add 50 µl (1.0X) of the resuspended AMPure XP beads and mix well on a vortex mixer or by pipetting up and down at least 10 times.
12. Incubate for 5 minutes at room temperature.
13. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), discard the supernatant that contains unwanted fragments **(Caution: do not discard the beads)**.
14. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
15. Repeat Step 14 once for a total of 2 washing steps.
16. Briefly spin the tube, and put the tube back in the magnetic rack.
17. Completely remove the residual ethanol, and air dry beads for 10 minutes while the tube is on the magnetic rack with the lid open.
18. Elute DNA target from the beads with 25 µl nuclease-free water. Mix well on a vortex mixer or by pipetting up and down, and put the tube in the magnetic rack until the solution is clear.
19. Without disturbing the bead pellet, transfer 23 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the NEBNext High-Fidelity 2X PCR Master Mix in the subsequent PCR step.

Perform PCR Library Enrichment

1 To the cDNA (23 µl) add the following components:

(blue) NEBNext High-Fidelity PCR Master Mix, 2X	25 µl
(blue) Universal PCR Primer (25 µM)	1 µl
(blue) Index (X) Primer (25 µM)*	1 µl
Total volume	50 µl

*If you are using the NEBNext Multiplex Oligos for Illumina (E7335 or E7500) for each reaction, only one of the 12 PCR primer indices is used during the PCR step.

Note: The Universal PCR primer and Index (X) Primer are contained in the NEBNext SinglePlex (NEB #E7350) or NEBNext Multiplex (NEB #E7335 or NEB #E7500) Oligos for Illumina.

2. PCR Cycling Conditions

CYCLE STEP	TEMPERATURE	TIME	CYCLES
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	
Annealing	65°C	30 seconds	12–15*, **
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. If 100 ng total RNA or 10 ng purified mRNA or ribosomal-depleted RNA are the starting input, it is recommended to perform 15 cycles of PCR.

** It is important to limit the number of PCR cycles to avoid overamplification. If overamplification occurs, larger molecular weight products (> 500 bp) will appear on the bioanalyzer trace.

Purify the PCR Reaction using Agencourt AMPure XP Beads

Note: X refers to the original sample volume from the above step.

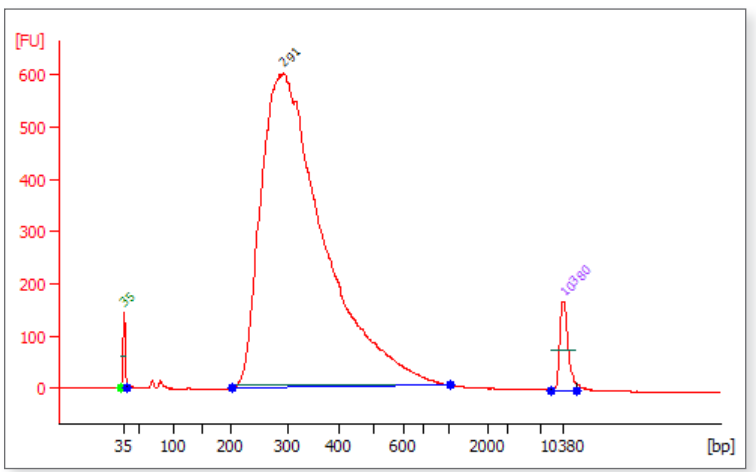
1. Vortex Agencourt AMPure XP Beads to resuspend.
2. Add 50 µl (1.0X) of resuspended Agencourt AMPure XP Beads to the PCR reaction (~ 50 µl). Mix well on a vortex mixer or by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube in a microcentrifuge and place the tube on an appropriate magnetic rack to separate beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.
5. Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once for a total of 2 washing steps.
7. Air dry the beads for 5 minutes while the tube is on the magnetic rack with the lid open.
8. Elute the DNA target from the beads into 23 µl nuclease free water. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and place it in the magnetic rack until the solution is clear.
9. Transfer 20 µl of the supernatant to a clean PCR tube, and store at –20°C.

Assess library quality on a Bioanalyzer® (Agilent high sensitivity chip).

1. Dilute (1:4) library in nuclease-free water.
2. Run 1 µl in a DNA High Sensitivity chip
3. Check that the electropherogram shows a narrow distribution with a peak size approximately 300 bp.

Note: If a peak at ~ 80 bp (primers) or 128 bp (adaptor-dimer) is shown in the bioanalyzer traces; Bring up the sample volume to 50 µl exactly with nuclease-free water and repeat the AMPure XP bead clean up step (steps 1–9 on page 11).

Figure 1: Example of RNA library size distribution on a Bioanalyzer.



Appendix A

Note: These recommendations have been optimized using Universal Human Reference Total RNA. Other types of RNA may require different fragmentation times.

Modified fragmentation times for longer RNA inserts.

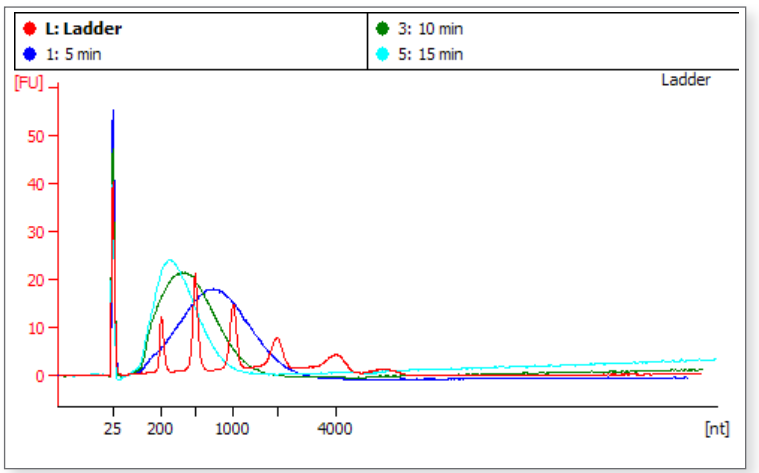


Figure 1: Bioanalyzer traces of RNA as shown in RNA Pico Chip. *mRNA isolated from Universal Human Reference RNA (1 µg) using the NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB #E7490) and Fragmented with First Strand Synthesis Reaction Buffer and Random Primer Mix (2X) at 94°C for 5, 10 or 15 minutes.* ***For libraries with RNA insert sizes larger than 300 bp, fragment RNA between 5–10 minutes.***

Table 1: Recommended size selection conditions for libraries with insert sizes larger than 300 bp.

LIBRARY PARAMETER	APPROXIMATE INSERT SIZE	250-400 bp	300-450 bp	400-600 bp	500-700 bp
	Approx. Final Library Size	350-500 bp	400-550 bp	500-700 bp	600-800 bp
BEAD VOLUME TO BE ADDED (µl)	1st Bead Selection	45	40	35	30
	2nd Bead Selection	20	20	15	15

Note: Any differences in insert sizes between the Agilent Bioanalyzer and that obtained from paired end sequencing can be attributed to the higher clustering efficiency of smaller sized fragments.

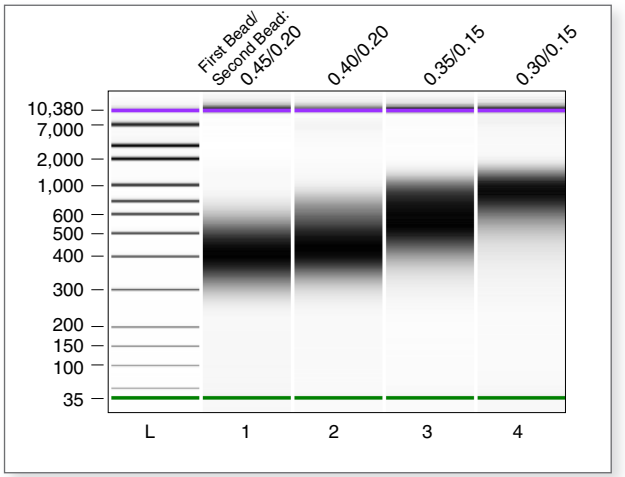
Size Selection of Adaptor-ligated DNA

⚠ ***For libraries with different size fragment inserts, refer to Table 1 for the appropriate volume of beads to be added. The size selection protocol is based on a starting volume of 100 µl. The protocol below is for libraries with a 300–450 bp insert size.***

1. Vortex AMPure XP beads to resuspend.
2. Adjust the final volume after ligation by adding nuclease free water for a 100 µl total volume.
3. Add 40 µl of resuspended AMPure XP beads to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times.
4. Incubate for 5 minutes at room temperature.
5. Quickly spin the tube and place the tube on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube **(Caution: do not discard the supernatant)**. Discard the beads that contain the unwanted large fragments.
6. Add 20 µl resuspended AMPure XP beads to the supernatant, mix well and incubate for 5 minutes at room temperature.
7. Quickly spin the tube and place it on an appropriate magnetic stand to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets **(Caution: do not discard beads).**
8. Add 200 µl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
9. Repeat Step 8 twice for a total of three washes.
10. Air the dry beads for 10 minutes while the tube is on the magnetic stand with the lid open.
11. Elute the DNA target from the beads into 28 µl of 10 mM Tris-HCl or 0.1 X TE, pH 8.0. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 23 µl to a new PCR tube for amplification.

Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the NEBNext High-Fidelity 2X PCR Master Mix in the subsequent PCR step.

Figure 2: Recommended size selection conditions for libraries with insert sizes > 300 bp.



RNA libraries made from Universal Human Reference Total RNA (500 ng) and size selected using different bead/DNA rations as indicated in Table 1. RNA was fragmented at 94°C for 5 minutes.

Troubleshooting Guide

OBSERVATIONS	POSSIBLE CAUSES	EFFECT	SUGGESTED SOLUTIONS
Presence of Bioanalyzer peaks <85 bp (Figure 1)	• Presence of Primers remaining after PCR clean up	Primers cannot cluster or be sequenced, but can bind to flowcell and reduce cluster density	• Clean up PCR again with 1.0X AMPure beads (second clean up may result in reduction of library yield)
Presence of 127 bp adaptor-dimer Bioanalyzer peak (Figure 1)	• Addition of non-diluted adaptor • RNA input was too low • RNA was over fragmented or lost during fragmentation • Inefficient Ligation	Adaptor-dimer will cluster and be sequenced. If ratio is low compared to library, may not be a problem but some reads will be dimers.	• Dilute adaptor (10 fold dilution) before setting up ligation reaction • Clean up PCR again with 1.0X AMPure beads (second clean up may result in reduction of library yield).
Presence of additional Bioanalyzer peak at higher molecular weight than the expected library size (~ 1,000 bp) (Figure 2)	• PCR artifact (over-amplification). Represents single-stranded library products that have self-annealed. If the PCR cycle number (or PCR input amount) is too high; in the late cycles of PCR the primers become limiting. Therefore, the adaptor sequences on either end of the fragment anneal to each other. This creates molecules with different insert sizes that run slower in the bioanalyzer.	If ratio is low compared to library, may not be a problem for sequencing	• Reduce number of PCR cycles.
Broad library size distribution (Figure 3)	• Under-fragmentation of the RNA	Library size will contain longer insert sizes	• Increase RNA fragmentation time

Figure 1:

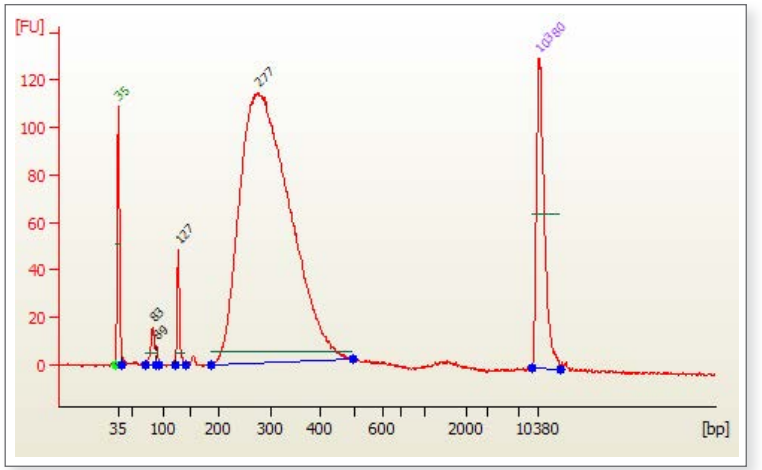


Figure 2:

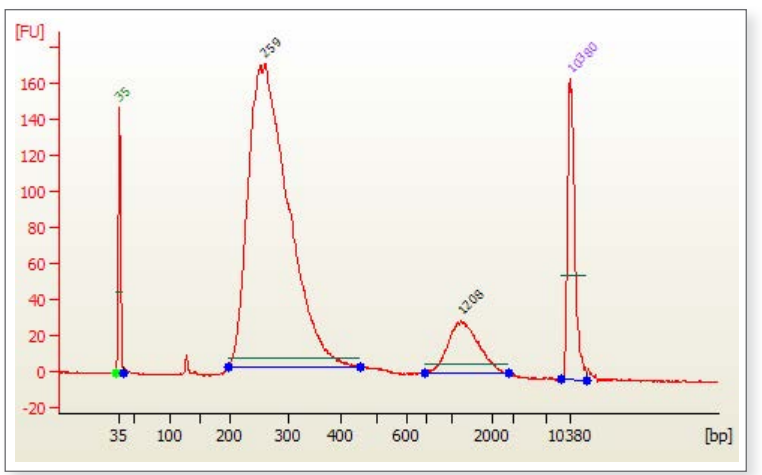
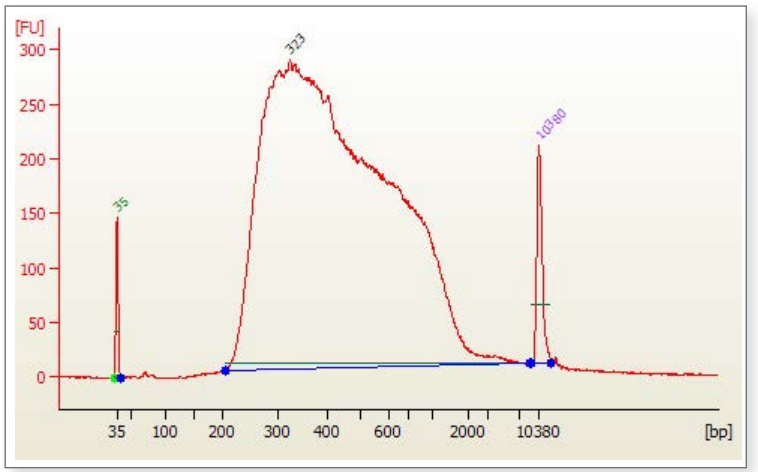


Figure 3:



Frequently Asked Questions (FAQs)

Q. What is the difference between the NEBNext Ultra Directional RNA library prep kit for Illumina (E7420) and the NEBNext Ultra RNA library prep kit for Illumina (E7530)?

A. The NEBNext Directional RNA library prep workflow preserves information about RNA strand orientation while the NEBNext Ultra RNA library prep does not. The NEBNext Ultra Directional RNA library prep contains dUTP in the second strand synthesis buffer that allows labeling the second strand cDNA and posterior excision with USER enzyme.

Q. What is the starting material I need to use when preparing libraries using the NEBNext Ultra Directional RNA kit?

A. The starting material is Total RNA (1ug-100 ng); previously isolated mRNA (10-100 ng) or Ribosomal-depleted RNA (10-100 ng).

Q. Where do I have to start the protocol if I have purified mRNA or Ribosomal-depleted RNA?

A. If starting material is purified mRNA or ribosomal-depleted RNA, proceed to (B)on page 6 of the manual.

Q. Which kit can I use to isolate Poly (A) mRNA from Total RNA?

A. To isolate poly (A) mRNA from Total RNA use the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490).

Q. Does the kit provide adaptor and primers?

A. No. Adaptors and primers are provided in the NEBNext Singleplex (NEB #E7350) or NEBNext Multiplex (NEB #E7335, #E7500) Oligos for Illumina.

NEBNext First Strand Synthesis Reaction Buffer

#E7421A: 0.192 ml **Concentration: 5X**
#E7421AA: 0.768 ml

Store at –20°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X First Strand Synthesis Reaction Buffer and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1X First Strand Synthesis Reaction Buffer and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a 10 µl reaction containing 1X First Strand Synthesis Reaction Buffer with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 1X First Strand Synthesis Reaction Buffer with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

Phosphatase Activity: Incubation of 1X First Strand Synthesis Reaction Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

NEBNext Second Strand Synthesis Reaction Buffer

#E7427A: 0.192 ml **Concentration: 10X**
#E7427AA: 0.768 ml

Store at –20°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1X NEBNext Second Strand Synthesis Reaction Buffer and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1X Second Strand Synthesis Reaction Buffer and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a 10 µl reaction containing 1X NEBNext Second Strand Synthesis Reaction Buffer with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 1X NEBNext Second Strand Synthesis Reaction Buffer with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

Phosphatase Activity: Incubation of 1X NEBNext Second Strand Synthesis Reaction Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

Random Primers

#E7422A: 0.048 ml
#E7422AA: 0.192 ml

Store at –20°C

Description: This mixture of random hexanucleotides is used to prime DNA synthesis *in vitro* along multiple sites of template RNA.

Sequence: 5´ d(N^6) 3´ [N=A,C,G,T]

Phosphorylated: No.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing 1 µl Random Primers and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 1 µl Random Primers and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a 10 µl reaction containing 1 µl Random Primers with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 1 µl Random Primers with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable RNase activity as determined by gel electrophoresis.

Phosphatase Activity: Incubation of 1 µl Random Primers in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

ProtoScript II Reverse Transcriptase

#E7423A: 0.024 ml **Concentration: 200,000 U/ml**
#E7423AA: 0.096 ml

Store at –20°C

Description: ProtoScript II Reverse Transcriptase is a recombinant M-MuLV reverse transcriptase with reduced RNase H activity and increased thermostability. It can be used to synthesize first strand cDNA at higher temperatures than the wild type M-MuLV. The enzyme is active up to 50°C, providing higher specificity, higher yield of cDNA and more full-length cDNA product up to 12 kb.

Source: The gene encoding a mutant M-MuLV Reverse Transcriptase (RNase H^-) is expressed in *E. coli* and purified to near homogeneity.

Supplied in: 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) IGEPAL® CA-630, 50% (v/v) glycerol

Quality Control Assays

16-Hour Incubation: A 50 µl reaction containing 1 µg of ϕX174 DNA and 100 units of ProtoScript II Reverse Transcriptase incubated for 16 hours at 37°C resulted in a DNA pattern free of detectable nuclease degradation as determined by agarose gel electrophoresis.

Exonuclease Activity: Incubation of a 50 µl reaction containing 100 units of ProtoScript II Reverse Transcriptase with 1 µg of a mixture of single and double-stranded [^{3}H] *E. coli* DNA (10^5 cpm/µg) for 4 hours at 37°C released < 0.2% of the total radioactivity.

RNase Activity: Incubation of a 10 µl reaction containing 100 units of ProtoScript II Reverse Transcriptase with 40 ng of RNA transcripts for 2 hours at 37°C resulted in no detectable degradation of the RNA as determined by gel electrophoresis.

Phosphatase Activity: Incubation of 100 units of ProtoScript II Reverse Transcriptase in protein phosphatase assay buffer containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Protein Purity (SDS-PAGE): ProtoScript II Reverse Transcriptase is > 95% pure as determined by SDS PAGE analysis using Coomassie blue detection.

Lot Controlled

Murine RNase Inhibitor

#E7424A: 0.015 ml
#E7424AA: 0.048 mll

Store at –20°C

Description: Murine RNase inhibitor is a 50 kDa recombinant protein of murine origin. The inhibitor specifically inhibits RNases A, B and C. It inhibits RNases by binding noncovalently in a 1:1 ratio with high affinity. It is not effective against RNase 1, RNase T1, S1 Nuclease, RNase H or RNase from *Aspergillus*. In addition, no inhibition of polymerase activity is observed when RNase Inhibitor is used with *Taq* DNA Polymerase, AMV or M-MuLV Reverse Transcriptases, or Phage RNA Polymerases (SP6, T7, or T3).

Recombinant murine RNase inhibitor does not contain the pair of cysteines identified in the human version that is very sensitive to oxidation, which causes inactivation of the inhibitor (1). As a result, murine RNase inhibitor has significantly improved resistance to oxidation compared to the human/porcine RNase inhibitors, even under conditions where the DTT concentration is low. Therefore, it is advantageous to use murine RNase inhibitor in reactions where high concentration DTT is adverse to the reaction (eg. Real-time RT-PCR).

Source: An *E. coli* strain that carries the Ribonuclease Inhibitor gene from mouse.

Supplied in: 20 mM HEPES-KOH, 50 mM KCl, 8 mM DTT and 50% glycerol.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing a minimum of 40 units of Murine RNase Inhibitor and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing a minimum of 40 units of Murine RNase Inhibitor and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Exonuclease Activity: Incubation of a 50 µl reaction containing 200 units of Murine RNase Inhibitor with 1 µg of a mixture of single and double-stranded [^{3}H] *E. coli* DNA (20^5 cpm/µg) for 4 hours at 37°C released < 0.5% of the total radioactivity.

Latent RNase Assay: Heating the Murine RNase Inhibitor for 20 minutes at 65°C, followed by incubation of a 10 µl reaction containing 40 units of RNase Inhibitor with 40 ng of RNA transcript for 4 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing 40 units of Murine RNase Inhibitor with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

Endonuclease Activity: Incubation of a 10 µl reaction containing 40 units of Murine RNase Inhibitor with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 40 units of Murine RNase Inhibitor in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

Reference:

1. Kim, B.M. et al. (1999). *Protein Science*, 8, 430–434.

NEBNext Second Strand Synthesis Enzyme Mix

#E7425A: 0.096 ml
#E7425AA: 0.384 ml

Store at –20°C

Description: NEBNext Second Strand Synthesis Enzyme Mix is optimized to convert of short single-stranded cDNAs to double-stranded cDNAs.

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analysis of this enzyme indicates > 95% enzyme purity.

Endonuclease Activity: Incubation of a 10 µl reaction containing 1 µl Second Strand Synthesis Enzyme Mix with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 1 µl Second Strand Synthesis Enzyme Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Functional Activity: One unit of the *E. coli* DNA Ligase ligated 50% of HindIII fragments of λ DNA (5´ DNA termini concentration of 0.12 µM, 300 µg/ml) in a total reaction volume of 20 µl in 30 minutes at 16°C in 1X *E. coli* DNA Ligase Reaction Buffer. One unit of *E. coli* DNA Polymerase I incorporated 10 nmol of dNTP into acid-insoluble material in a total reaction volume of 50 µl in 30 minutes at 37°C in 1X EcoPol Reaction Buffer with 33 µM dNTPs including [^{3}H]-dTTP and 70 µg/ml denatured herring sperm DNA. Incubation of 50 units of RNase H with 1 µg sonicated and denatured [^{3}H]-DNA (10^5 cpm/µg) for 30 minutes at 37°C in 50 µl reaction buffer released < 0.1% radioactivity.

Lot Controlled

Reference:

1. Gubler et al. (1983). *Gene* 25, 263–269.

NEBNext End Prep Enzyme Mix

#E7371A: 0.072 ml
#E7371AA: 0.288 ml

Store at –20°C

Description: NEBNext End Prep Enzyme Mix is optimized to convert 5 ng–1 µg of fragmented DNA to repaired DNA having 5´-phosphorylated dA-tailed ends.

Quality Control Assays

SDS-PAGE Purity: SDS-PAGE analyses of each individual enzyme indicates > 95% enzyme purity.

Endonuclease Activity: Incubation of a minimum of 10 µl of this enzyme mix with 1 µg of ϕX174 RF I DNA in assay buffer for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of a minimum of 10 µl of this enzyme mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Functional Activity (Nucleotide Incorporation, Phosphorylation and dA-Tailing): 1 µl of this enzyme mix repairs and phosphorylates the ends of > 95% of 0.5ug of DNA fragments containing both 3´ and 5´ overhangs with 20 minutes at 25°C, in 1X End Repair Reaction buffer, as determined by capillary electrophoresis.

Lot Controlled

NEBNext End Repair Reaction Buffer

#E7372A: 0.156 ml
#E7372AA: 0.624 ml

Concentration: 10X

Store at –20°C

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of this reaction buffer at a 1X concentration with 1 µg of ϕX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this reaction buffer at 1X concentration with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Phosphatase Activity: Incubation of this reaction buffer at a 1X concentration in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

Blunt/TA Ligase Master Mix

#E7373A: 0.360 ml
#E7373AA: 0.720 ml (2 vials provided)

Store at –20°C

Description: Blunt/TA Ligase Master Mix is a ready-to-use solution of T4 DNA Ligase, proprietary ligation enhancer, and optimized reaction buffer.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing this reaction buffer at 1X concentration and 1 µg T3 DNA incubated for 16 hours at 37°C also results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of this reaction buffer at a 1X concentration with 1 µg of ϕX174 RF I DNA for 4 hours at 37°C in 50 µl reactions results in less than 10% conversion to RF II as determined by agarose gel electrophoresis.

RNase Activity: Incubation of this reaction buffer at 1X concentration with 40 ng of a FAM-labeled RNA transcript for 16 hours at 37°C results in no detectable RNase activity as determined by polyacrylamide gel electrophoresis.

Transformation Assay: LITMUS™ 28 vector is cut with EcoRV (blunt), treated with calf intestinal phosphatase and gel purified. Blunt inserts from a HaeIII digest of ϕX174 DNA are ligated into the vector at a 3:1 insert:vector ratio using the Blunt/TA Ligase Master Mix Protocol. Ligation products are transformed as described.

Each lot exceeds the following standards:

	Efficiency (transformants/µg)	
	Recircularization	Insertion
Blunt ends	> 1×10^7	> 2.5×10^6
Uncut vector	> 1×10^8	

Lot Controlled

Nuclease-free Water

#E7431A: 8 ml
#E7431AA: 30 ml

Store at –20°C or 4°C

Description: Nuclease-free Water is free of detectable DNA and RNA nucleases and phosphatases and suitable for use in DNA and RNA applications.

Quality Control Assays

16-Hour Incubation: 50 µl reactions containing Nuclease-free Water and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing Nuclease-free Water and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Endonuclease Activity: Incubation of a 10 µl reaction containing Nuclease-free Water with 1 µg of ϕX174 RF I supercoiled DNA for 4 hours at 37°C results in < 10% conversion to RF II (nicked molecules) as determined by gel electrophoresis.

RNase Activity: Incubation of a 10 µl reaction containing Nuclease-free Water with 40 ng of RNA transcript for 16 hours at 37°C resulted in no detectable degradation of RNA as determined by gel electrophoresis.

Phosphatase Activity: Incubation of 1X Second Strand Synthesis Reaction Buffer in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Lot Controlled

NEBNext High-Fidelity 2X PCR Master Mix

E7375A: **0.6 ml** **Concentration: 2X**
E7375AA: **1.2 ml (2 vials provided)**

Store at –20°C

Description: The NEBNext High-Fidelity 2X PCR Master Mix is specifically optimized for robust, high-fidelity amplification of next-generation sequencing (NGS) libraries, regardless of GC content. The polymerase component of the master mix, Q5® High-Fidelity DNA Polymerase, is a novel thermostable DNA polymerase that possesses 3´→ 5´ exonuclease activity, and is fused to a processivity-enhancing Sbcso7d domain. Q5 High-Fidelity DNA Polymerase also has an ultra-low error rate (> 50-fold lower than that of *Taq* DNA Polymerase and 6-fold lower than that of *Pyrococcus furiosus* (Pfu) DNA Polymerase)

Quality Control Assays

16-Hour Incubation: A 50 µl reactions containing NEBNext High-Fidelity 2X PCR Master Mix and 1 µg of HindIII digested Lambda DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis. 50 µl reactions containing 100 units of NEBNext High-Fidelity 2X PCR Master Mix and 1 µg of T3 DNA incubated for 16 hours at 37°C results in no detectable non-specific nuclease degradation as determined by agarose gel electrophoresis.

Phosphatase Activity: Incubation of NEBNext High-Fidelity 2X PCR Master Mix in protein phosphatase assay buffer (1 M diethanolamine @ pH 9.8 and 0.5 mM $MgCl_2$) containing 2.5 mM *p*-nitrophenyl phosphate at 37°C for 4 hours yields no detectable *p*-nitrophenylene anion as determined by spectrophotometric analysis at 405 nm.

Functional Activity PCR: 30 cycles of PCR amplification of 20 ng genomic DNA in a 50 µl reaction containing 0.5 µM primers and 1X NEBNext High-Fidelity PCR Master Mix result in the expected 737 bp product.

Lot Controlled

Revision History

Revision #	Description
2.0	Added RNA input recommendations, removed the size selection for 200 bp fragments - replaced with clean up step. Added additional recommendation for larger insert sizes (Appendix A), Troubleshooting Guide, FAQs. Removed additional washing step in PolyA Isolation Protocol. Moved stopping point from after Second Strand cDNA Synthesis to follow the clean up step.Changed First Strand cDNA Synthesis conditions from 50 minutes at 42°C to 15 minutes at 42°C. Added recommendation to dilute the NEBNext adaptor.

USA
New England Biolabs, Inc.
240 County Road
Ipswich, MA 01938-2723
Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227
Toll Free: (USA Tech) 1-800-632-7799
Fax: (978) 921-1350
e-mail: info@neb.com
www.neb.com

CANADA
New England Biolabs, Ltd.
Telephone: (905) 665-4632
Toll Free: 1-800-387-1095
Fax: (905) 665-4635
Fax Toll Free: 1-800-563-3789
e-mail: info.ca@neb.com
www.neb.ca

CHINA, PEOPLE'S REPUBLIC
New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com
www.neb-china.com

FRANCE
New England Biolabs France
Free Call: 0800-100-632
Free Fax: 0800-100-610
e-mail: info.fr@neb.com
www.neb-online.fr

GERMANY & AUSTRIA
New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Free Call: 00800/246 52277 (Austria)
Fax: +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info.de@neb.com
www.neb-online.de

JAPAN
New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax: +81 (0)3 5669 6192
e-mail: info@neb-japan.com
www.nebj.jp

SINGAPORE
New England Biolabs Pte. Ltd.
Telephone: +65 6776 0903
Fax: +65 6778 9228
e-mail: sales.sg@neb.com
www.neb.sg

UNITED KINGDOM
New England Biolabs (UK) Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info.uk@neb.com
www.neb.uk.com

NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

Version 2.0 7/13