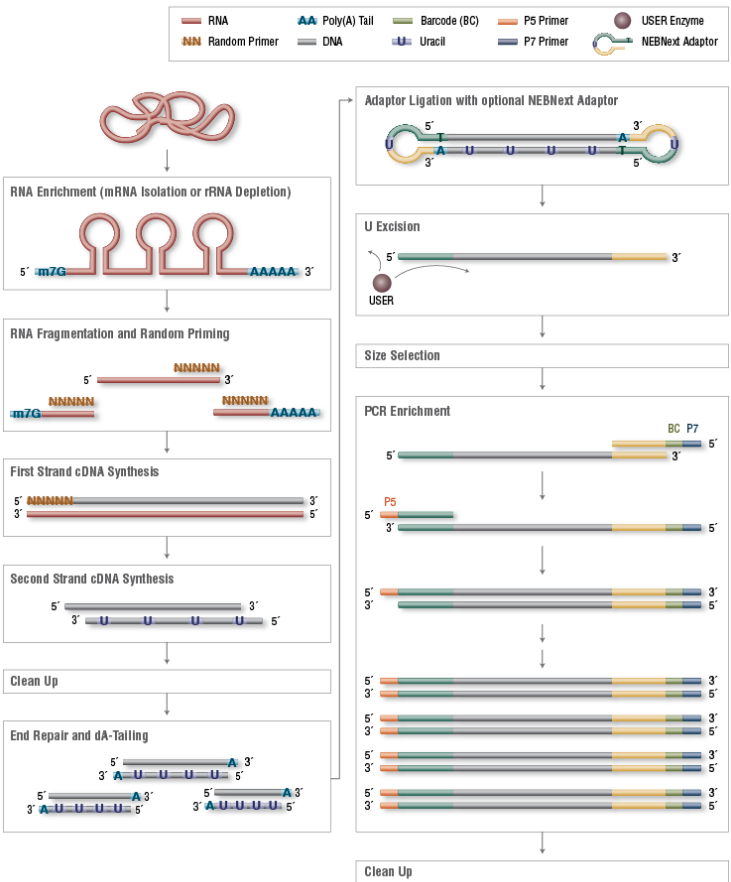
NEBNext Protocol (E7420)
Second Day



v8.0

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

This protocol has been optimized using Universal Human Reference Total RNA.

3.1 RNA Fragmentation, Priming and First Strand cDNA Synthesis

RNA fragmentation is only required for intact or partially degraded RNA. Recommended fragmentation times can be found in Table 3.1. Follow protocol in 3.1A to set up the reaction. For highly degraded RNA (FFPE Samples) which do not require fragmentation proceed to Step 3.1B.

3.1A RNA Fragmentation and Priming Starting from Intact or Partially Degraded RNA:

1. Set up the following reaction and mix by gentle pipetting:

Purified mRNA or Ribosome 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

2. Incubate the sample at 94°C following the recomendations in Table 3.1 for fragments sizes ~200 nt.

Table 3.1. Suggested fragmentation times based on RIN number of RNA input.

RNA TYPE	RIN	FRAG. TIME
Intact RNA	>7	15 min. at 94°C
Partially Degraded RNA	2–6	7–8 min. at 94°C

Refer to Appendix A for fragmentation conditions if you are preparing libraries with large inserts (> 200 bp). Conditions in Appendix A only apply for intact RNA.

3. Transfer the tube to ice.

First Strand cDNA Synthesis

Dilute Actinomycin D stock solution (5 µg/µl) to 0.1 µg/µl in nuclease free water for immediate use.

Note: Dilute solutions of Actinomycin D are very sensitive to light. In solution, Actinomycin D tends to adsorb to plastic and glass. For these reasons, unused dilute solutions should be discarded and not stored for further use. However, frozen aliquots of a concentrated stock solution (5 µg/µl) in DMSO are expected to be stable for at least a month at –20°C.

4. To the fragmented and primed mRNA from Step 3 in Section 3.1A (10 µl) add the following components and mix by gentle pipetting:

(pink) Murine RNase Inhibitor	0.5 µl
Actinomycin D (0.1 µg/µl)	5 µl
(pink) ProtoScript II Reverse Transcriptase	1 µl
Nuclease free water	3.5 µl
Final volume	20 µl

Note: If you are following recommendations in Appendix A, for longer RNA fragments, increase the incubation at 42°C from 15 minutes to 50 minutes in Step 5.

5. Incubate the sample in a preheated thermal cycler (with the heated lid set at 105°C) as follows:

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

6. Proceed directly to Second Strand cDNA Synthesis, Section 3.2.

3.2 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.

3.3 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.

Sodium Azide Waste!!

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 lid open.

   Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnet. Elute the DNA target from the beads into 60 µl 0.1X TE Buffer or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
9. Remove 55.5 µl of the supernatant and transfer to a clean eppendorf tube with “cDNA” sticker label

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

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NEBNext Protocol

Third Day

3.4 Perform End Prep of cDNA Library

1. Mix the following components in a sterile nuclease free tube:

Purified double-stranded cDNA (Step 9, Section 2.7)	55.5 µl
(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 (with the heated lid set at 75°C) as follows:
   30 minutes at 20°C
   30 minutes at 65°C
   Hold at 4°C

3. Proceed immediately to Adaptor Ligation.

3.5 Perform Adaptor Ligation

Dilute the (red) NEBNext Adaptor* for Illumina (15 µM) to 1.5 µM with a 10-fold dilution (1:9) with 10 mM Tris-HCl and 10 mM NaCl for immediate use.

1. Add the following components directly to the End Prep Reaction **(Caution: Do not pre-mix the components to prevent adaptor-dimer formation):**

End Prep Reaction	65 µl
(red) Blunt/TA Ligase Master Mix	15 µl
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, #E7710, #E7730, #E6609 or #E7600) 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.

⚠ ***A precipitate can form upon thawing of the NEBNext Hot Start HiFi PCR Master Mix. To ensure optimal performance, place the master mix at room temperature while purifying the ligation reaction. Once thawed, gently mix by inverting the tube several times.***

3.6 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, Chapter 4.**

1. Add nuclease-free water to the ligation reaction to bring the reaction volume to 100 µl. It is important to ensure the final volume is 100 µl prior to adding AMPure XP Beads.

   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)**.

   Sodium Azide Waste!!
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 5 minutes while the tube is on the magnetic rack with the lid open.

   Caution: Do not over dry the beads. This may result in lower recovery of DNA target.

9. Remove the tube from the magnet. Elute DNA target from the beads with 52 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. 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 5 minutes while the tube is on the magnetic rack with the lid open.

    Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
18. Remove the tube from the magnet. Elute DNA target from the beads with 19 µl 0.1X TE or 10 mM Tris-HCl. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature. Put the tube in the magnetic rack until the solution is clear.
19. Without disturbing the bead pellet, transfer 17 µl of the supernatant to a clean PCR tube and proceed to PCR enrichment.

3.7 PCR Enrichment of Adaptor Ligated DNA

3.7B PCR Library Enrichment

1. To the cDNA (17 µl) from Step 19 Section 3.6 add the following components and mix by gentle pipetting:

(blue) NEBNext USER Enzyme	3 µl
(blue) NEBNext Q5 Hot Start HiFi PCR Master Mix	25 µl
(blue) Index/ Universal Primer Mix*	5 µl
Total volume	50 µl

* The primers are provided in NEBNext Multiplex Oligos for Illumina, NEB #E6609. Please refer to the NEB #E6609 manual for valid barcode combinations and tips for setting up PCR reactions.

2. PCR Cycling Conditions

CYCLE STEP	TEMP	TIME	CYCLES
USER Digestion	37°C	15 minutes	1
Initial Denaturation	98°C	30 seconds	1
Denaturation Annealing/Extension	98°C 65°C	10 seconds 75 seconds	12–15*, **
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

* The number of PCR cycles should be adjusted based on RNA input. If 10 ng enriched RNA is 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.

3. Proceed to Section 3.8 (Purify the PCR Reaction using Agencourt AMPure XP Beads).

3.8 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 45 µl (0.9X) 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.

Sodium Azide Waste!!

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.

   Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
8. Remove the tube from the magnetic rack. Elute the DNA target from the beads into 23 µl 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, quickly spin the tube in a microcentrifuge and incubate for 2 minutes at room temperature. Place the tube in the magnetic rack until the solution is clear.
9. Transfer 20 µl of the supernatant to a clean l

   eppendorf tube with “fin-D” sticker label and store at -20 C

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.
2.1	Renamed "elution buffer" in text to "Tris Buffer".
3.0	Added protocol for use with NEB #E6310. Changed NEBNext adaptor dilution recommendations. Changed AMPure Bead drying time to 5 minutes. Updated final library elution and dilution for Bioanalyzer to 10 mM Tris or 0.1X TE. Changed ratio of AMPure Beads to 0.9X in final clean up after PCR reaction.
4.0	Included protocol for use with NEBNext Q5 Hot Start HiFi PCR Master Mix. Include protocol for changes in concentration of NEBNext Singleplex and Multiplex Oligos for Illumina. Changed all AMPure Bead elutions to 0.1X TE or 10 mM Tris-HCl. Added 2 minute incubation after eluting DNA from AMPure Beads.
5.0	Removed protocol for use with NEBNext High-Fidelity 2X PCR Master Mix. Include protocol for use with NEBNext Multiplex Oligos (96 Index Primers, NEB #E6609).
6.0	Volume of beads increased from 15 µl to 20 µl. Additional mixing and incubation steps were added after each thermocycler incubation. Elution volume changed before fragmentation. Updated table on page 40.
7.0	Component change: The name, part # and formulation of RNase H has changed.
8.0	Protocol updated to include NEB #E7710 and NEB #E7730. Correction of typos and clarifications in several places. Section C in the PCR setup step was removed because all of the 25 µM primers are now expired.