firefly®+ technical note

# NEBNext UltraExpress® RNA Library Prep-rRNA depletion

## Overview

- This technical note provides supporting information for automating NEBNext UltraExpress® RNA Library Prep on SPT Labtech firefly liquid handler. These protocols are available to download from the firefly community. Here, we outline protocol run times, parts required and provide details on the steps performed in each protocol.
- This protocol performs workflow described in the NEBNext rRNA Depletion Kits (NEB #E7400, #E7405, #E7850, #E7860) and NEBNext UltraExpress RNA Library Prep Kit (NEB #E3330) (Version 4.0_10/25).
- Deviation from the NEB manual:
  - resuspend rRNA depleted RNA samples in 7 µL Fragmentation master mix, instead of 6.5 µL.
  - 0.65x Beads ratio for phased beads cleanup after the PCR amplification.
  - The Index anchor primers were diluted 150 times.
  - Decreasing the fragmentation time from 15 to 8 minutes resulted in an average fragment size of approximately 320 bp.
- The whole workflow from rRNA depletion to NGS library cleanup is split into two protocols on firefly+ with on deck thermo cycler. No user interaction needed during the protocol operation. Variables are setup for NUMBER OF COLUMNS and INDEX PCR cycles.
- Instrument configuration: firefly plus 6-head, genomics, ATL head, 96 ODTC.
- This protocol has been written to be compatible with v2.0.3 and tested for samples of 250 ng RNA samples with different quality or 20-240 ng Universal Human Reference RNA (UHRR) samples and 13 PCR cycles were used.

## Acknowledgements

The protocol was developed and validated at our collaboration partner site, the Genomics Core Facility at the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany. We would like to extend our sincere thanks to Vivianne Niehaus, M.Sc., and Ferris Jung for their meticulous work, dedication, and invaluable contributions to this project. We also gratefully acknowledge New England Biolabs for generously providing the reagents used for testing.

## firefly protocols

| Protocol number | Protocol name | firefly+ run time (minutes) |
|---|---|---|
| Protocol 1 of 2 | NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 1 | 300 |
| Protocol 2 of 2 | NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2 | 200 |

Table 1. protocols & estimated run times used in NEBNext UltraExpress® RNA Library Prep rRNA depletion workflow on firefly+ for 24 samples.



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# Input variables

| NEBNext UltraExpress® RNA Library Prep - rRNA depletion | | | | |
|---|---|---|---|---|
| **Protocol** | **Input variables** | **Interval** | **Minimal** | **Maximal** |
| Protocol 1 of 2 | Number of columns | 1 | 1 | 12 |
| Protocol 2 of 2 | Number of columns | 1 | 1 | 12 |
| Protocol 2 of 2 | Index PCR cycles | File variable | | |

Table 2. Variables used in NEBNext UltraExpress® RNA Library Prep rRNA depletion workflow on firefly+.

# Reagent volumes

The reagent volumes required to run NEBNext UltraExpress RNA Library Prep -rRNA depletion on SPT Labtech firefly+ depend on the number of samples being processed. Default required minimum volumes for these reagents, based on the number of samples shown in the Input variables table, are shown below and in the execute section of the firefly software.

## Protocol 1 of 2

| NEBNext UltraExpress® RNA Library Prep - rRNA depletion Part 1 | |
|---|---|
| **Reagent in reservoir** | **Total required volume (µL)** |
| Fragmentation MM | 243 |
| Second Strand Synthesis MM | 795 |
| DNase 1 MM | 795 |
| **Reagent in a single column in reagent plate** | **Total required volume (µL)** |
| First Strand Synthesis MM | Number of columns x 5 +2 µL/ well in 1 column |
| RNase H MM | Numberof columns x 5 +3 µL/ well in 1 column |
| Probe Hybridization hybridization MM | Number of columns x 4 +3 µL/ well in 1 column |
| **Reagent in reagent plate** | **Total required volume (µL)** |
| Ethanol | 800 µL/well x Number of columns |
| 0.1X TE | 130 µL/well x Number of columns |
| SPRI Beads | 180 µL/well x Number of columns |

Table 3. NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 1minimum required reagent volumes for 24 samples in 3 columns.

## Protocol 2 of 2

| NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2 | |
|---|---|
| **Reagent in reservoir** | **Total required volume (µL)** |
| Diluted adaptor | 130 |
| Ligation MM | 363 |
| MSTC MM | 1275 |
| Reagent in a single column in reagent plate | Total required volume (µL) |
| End Prep Reaction MM | Number of columns x 4 +3 µL/ well in 1 column |
| USER enzyme | Number of columns x 3 +5 µL/ well in 1 column |
| **Reagent in reagent plate** | **Total required volume (µL)** |
| Ethanol | 1000 µL/well x Number of columns |
| 0.1X TE | 100 µL/well x Number of columns |
| SPRI Beads | 160 µL/well x Number of columns |
| Beads reconstitute buffer | 45 µL/well x Number of columns |
| Indexes | 12 µL/well x Number of columns |

Table 4. NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2 minimum required reagent volumes for 24 samples in 3 columns.

# Protocol overview

## Protocol 1 of 2

## NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 1




Figure 1. Schematic illustration of the workflow enabled in Protocol NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 1 on firefly+. rRNA is depleted, fragmented and converted into double-stranded cDNA through reverse transcription and cleanup steps.

This protocol performs the following sections described in the NEBNext rRNA Depletion Kits (NEB #E7400, #E7405, #E7850, #E7860) and UltraExpress RNA Library Prep Kit (NEB #E3330) (Version 4.0_10/25).

- rRNA depletion and fragmentation
- First Strand cDNA Synthesis
- Second Strand cDNA Synthesis
- Purification of double-stranded cDNA

Prior to executing this protocol:

- NEBnext UltraExpress RNA Library Prep -rRNA depletion part 1 requires a pre-filled RNA sample input plate containing 11 µL RNA samples (25-250 ng).
- RNase H MM, Probe Hybridization MM and First Strand Synthesis MM are loaded in a single column in a reagent plate on a pre-cooled Thermo block.
- The following reagents need to be prepared in reagent plates in all wells according to Number of columns
  - Ethanol: 800 µL/well x Number of columns
  - 0.1X TE: 130 µL/well x Number of columns
  - SPRI Beads: 180 µL/well x Number of columns

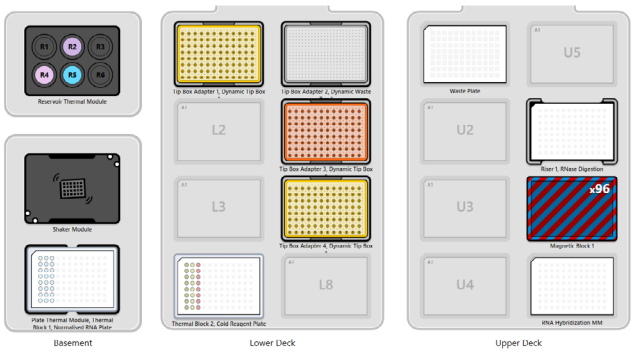



Figure 2. Deck layout for preparation of 24 samples with protocol NEBNext UltraExpress® RNA Library Prep -Part 1 on firefly+.

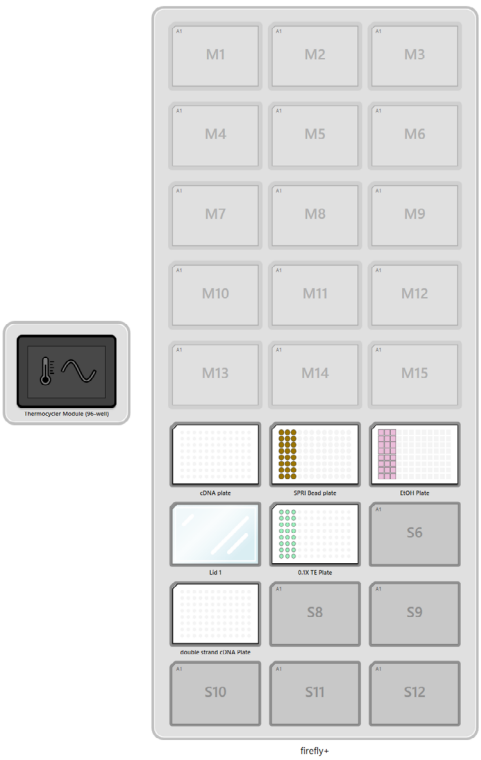



Figure 3. Plus deck layout for preparation of 24 samples with protocol NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 1 on firefly+.

## Protocol 2 of 2

## NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2




Figure 4. Schematic illustration of the workflow enabled in Protocol NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2 on firefly+. Double-stranded cDNA is end-repaired, adapter-ligated, purified, amplified, and cleaned up to produce a sequencing-ready DNA library

This protocol performs the following sections described in the NEBNext rRNA Depletion Kits (NEB #E7400, #E7405, #E7850, #E7860) and UltraExpress RNA Library Prep Kit (NEB #E3330) (Version 4.0_10/25).

- End Prep of cDNA library
- Adaptor Ligation
- PCR Enrichment of Adaptor Ligated DNA
- Phased Bead Cleanup of PCR Reaction

Prior to executing this protocol:

- NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2 protocol requires a pre-filled cDNA sample input plate containing 20 µL cDNA samples prepared from Part 1.
- NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2 protocol requires Index plate with 12 µL Indexes
- USER Enzyme and End Prep Reaction MM are loaded in a single column in a reagent plate on a pre-cooled thermo block, which will be aliquoted in the protocol.
- The following reagents need to be prepared in reagent plates in all wells according to number of columns:
  - Ethanol: 1000 µL/well x Number of columns
  - 0.1X TE: 100 µL/well x Number of columns
  - SPRI Beads: 160 µL/well x Number of columns
  - Bead Reconstitution Buffer: 45 µL/well x Number of columns

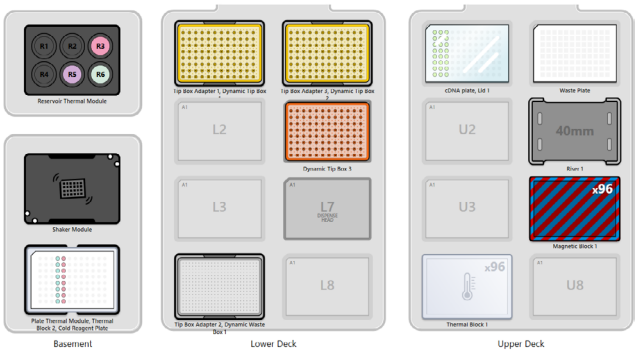



Figure 5. Deck layout for preparation of 24 samples with protocol NEBNext UltraExpress RNA Library Prep -rRNA depletion Part 2 on firefly+.

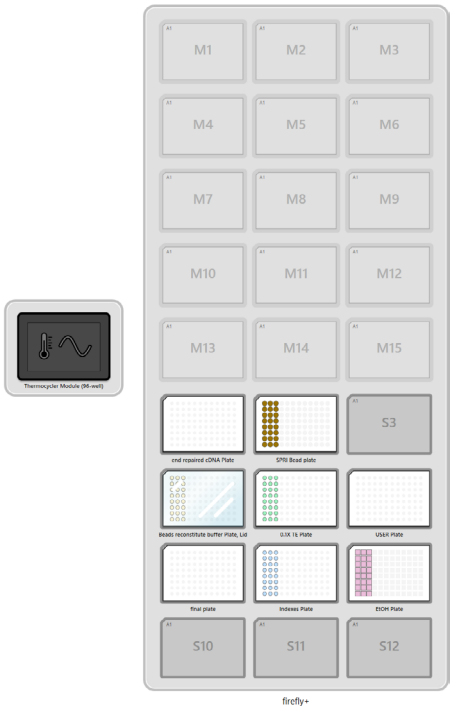



Figure 6. Plus deck layout for preparation of 24 samples with protocol NEBNext UltraExpress® RNA Library Prep -rRNA depletion Part 2 on firefly+.

# Appendix:

## Consumables and accessories

| Supplier | Part name | Part number | Part 1 | Part 2 |
|---|---|---|---|---|
| SPT Labtech | AT-Load firefly® Pipette Tips, 100 µL with filters, Sterile 96 Tips Per Rack | 125-096-FF-AL-FS | 2 | 2 |
| SPT Labtech | AT-Load firefly® Pipette Tips, 35 µL with filters, Sterile 96 Tips Per Rack | 50-096-FF-AL-FS | 1 | 1 |
| SPT Labtech | dragonfly® discovery Sterile, Ultra Low Retention Syringes | 4150-07209 | 1 | 0 |
| SPT Labtech | dragonfly® discovery Sterile Syringes | 4150-07201 | 2 | 3 |
| SPT Labtech | Reservoir | Low dead volume: 4150-07203 (sterile); 4150-07203<br>Standard: 4150-07204 (sterile); 4150-07103 | 3 | 3 |
| SPT Labtech | Empty Tip Box | NA (not applied) | 1 | 1 |
| SPT Labtech | 40mm Upper Deck Riser | 3276-01838 | 1 | 1 |
| SPT Labtech | ATL 35-125uL Tip Stand | 3276-08335 (REV 2) | 4 | 3 |
| SPT Labtech | Thermo Adapter Block | 3276-01065 | 2 | 2 |
| Eppendorf | twin.tec PCR | 30128648 | 7 | 8 |
| Corning | Axygen round-bottom 2.2mL deep well | P-2ML-SQ-C-S | 1 | 1 |
| Corning | Axygen V-bottom | P-96-459V-C | 1 | 1 |
| Thermo Fisher Scientific | Abgene 1.2mL Deep Well | AB-1127 | 1 | 1 |
| Alpaqua Engineering | Alpaqua Magnum FLX | A000400 | 1 | 1 |
| Hamilton | PCR ComfortLid | 814300 | 1 | 1 |
| Azenta | TD-universal-microplate-lid | 4TI-0290 | 0 | 1 |

Table 5. Consumables and labware required for NEBNext UltraExpress® RNA Library Prep rRNA depletion on firefly+.

For questions regarding SPT Labtech firefly, contact our support team through **fireflysupport@sptlabtech.com**

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