firefly® technical note

NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina

This technical note provides supporting information for automation of the NEBNext Ultra II FS DNA Library Prep kit on firefly. The firefly protocols are listed below and 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.

Overview

The NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina is optimized for the construction of high quality, high yield libraries using a broad range of DNA input amounts. The library preparation workflow uses NEBNext FS (Fragmentation System) enzymatic fragmentation reagents, eliminating the need to mechanically shear the DNA input. The FS workflow uses a single enzyme mix to perform the fragmentation, end repair and dA-tailing, which greatly simplifies the workflow.

The firefly protocols are based on the method described in the NEB instruction manual for kits E7805 and E6177 (v4.0_7/23) and the firefly protocol names reflect the section numbering used in the instruction manual.

firefly protocols

Protocol number	Protocol name
Protocol 1 of 4	1.1-1.2 Fragmentation, End Prep and Adaptor Ligation
Protocol 2 of 4	1.3 Adaptor Clean-up
Protocol 3 of 4	1.4 PCR Enrichment
Protocol 4 of 4	1.5 PCR Clean-up
Optional protocol	2.3 Size Selection for DNA Input ≥100ng

Table 1. firefly protocols used to run the NEBNext Ultra II FS DNA Library Prep Kit for Illumina

Workflow overview

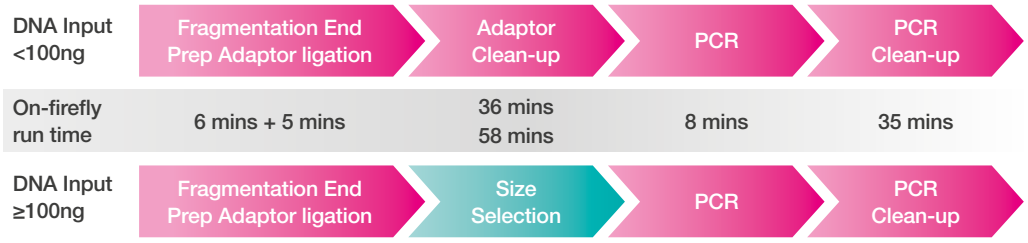

Figure 1. Workflow and firefly run times for the NEBNext Ultra II FS DNA Kit. Where the PCR reaction setup takes place on firefly and the PCR reaction takes place on an off-deck thermocycler.

Key workflow features

- Enzymatic shearing of gDNA
- Fragmentation and End prep (end repair + dA-tailing) are performed in one reaction
- NEBNext adaptors are used – these adaptors have a hairpin loop structure that contains a uracil base
  - Uracil is removed by an enzyme mix (USER) to open the loop and enable PCR
- USER/PCR reagent dispenses and incubations are combined
  - Minimizes the dead volume of USER required by the protocol
  - Reduces the number of operator touch-points in the protocol

Deviations from the standard manual protocol

- The dispense and incubation of USER enzyme and PCR mastermix has been combined. 28µL of a master mix consisting of the USER enzyme and Q5 Master Mix delivers the equivalent of 3µL USER enzyme + 25µL Q5 Master Mix to each sample. The USER incubation and PCR incubation are combined in the same thermocycler program, see below for details.
- The input volume into the adaptor clean-up is 68.5µL rather than 71.5µL, which reduces the bead volume for the 0.8X adaptor clean-up from 57µL to 55µL.
- The volume of ethanol used for each bead clean-up wash is 60µL per well rather than 200µL per sample.

The firefly protocols (listed in Table 1) are set up to process 96 samples per run. Protocols can be adapted to run with less than 96 samples – your dedicated Applications Scientist can assist with this.

We recommend that one 96-sample kit is used to process 96-samples or 2 x 48-sample runs only.

Reagent volumes and master mixes

Tables 2 and 3 below outline the reagent volumes the dispense head uses in each firefly protocol, to perform one 96 sample run and two 48 sample runs respectively. If fewer libraries are to be prepared, the number of columns to be processed can be adjusted as a variable in the firefly protocol, which will update the volumes required in each reservoir and will be displayed in the Execute screen of the firefly software.

Reservoirs are filled and loaded onto firefly immediately before starting each protocol.

To minimize the volume of each reagent used, master mixes (consisting of multiple constituent reagents) are combined directly in the dispense head reservoir and are manually tip-mixed in the reservoir before use. When tip-mixing, care is needed to prevent bubbles from forming.

Protocol	Protocol name	Reservoir	Reagent name	Total volume (uL)	Reservoir type	Constituent reagents	Volume of each constituent (uL)
1 of 4	1.1-1.2 Fragmentation, End Prep and Adaptor Ligation	R1	Adaptor - diluted as required	320	LDV		
		R2	FS Enzyme mix	944	LDV	FS Reaction Buffer	734
						FS Enzyme Mix	210
		R4	Ligation Master Mix + Enhancer	3316	Standard	Ligation Master Mix	3209
						Enhancer	107
2 of 4	1.3 Adaptor Clean-up	R1	Beads	5520	Standard		
		R2, R3, R5, R6	80% Ethanol	40000	Standard		
		R4	0.1X TE	2077	Standard		
3 of 4	1.4 PCR Enrichment	R4	NEBNext Ultra II Q5 Master Mix + USER enzyme	2928	Standard	Q5 Master Mix	2614
						USER Enzyme	314
4 of 4	1.5 PCR Clean-up	R1	Beads	4819	Standard		
		R2, R3, R5, R6	80% Ethanol	20000	Standard		
		R4	0.1X TE	4272	Standard		

Table 2. Dispense head reagent volumes used for processing 96 samples. A 100µL reservoir overfill volume is included in the Total volume for the Ligation Master Mix + Enhancer reagent.

Protocol	Protocol name	Reservoir	Reagent name	Total volume (uL)	Reservoir type	Constituent reagents	Volume of each constituent (uL)
1 of 4	1.1-1.2 Fragmentation, End Prep and Adaptor Ligation	R1	Adaptor - diluted as required	200	LDV		
		R2	FS Enzyme mix	512	LDV	FS Reaction Buffer	398
						FS Enzyme Mix	114
		R4	Ligation Master Mix + Enhancer	1583	LDV	Ligation Master Mix	1532
						Enhancer	51
2 of 4	1.3 Adaptor Clean-up	R1	Beads	2880	Standard		
		R2, R3, R5, R6	80% Ethanol	40000	Standard		
		R4	0.1X TE	1213	Standard		
3 of 4	1.4 PCR Enrichment	R4	NEBNext Ultra II Q5 Master Mix + USER enzyme	1419	LDV	Q5 Master Mix	1414
						USER Enzyme	170
4 of 4	1.5 PCR Clean-up	R1	Beads	2530	Standard		
		R2, R3, R5, R6	80% Ethanol	20000	Standard		
		R4	0.1X TE	2256	Standard		

Table 3. Dispense head reagent volumes for processing 48 samples. A 20µL reservoir overfill volume is included in the Total volume for the Ligation Master Mix + Enhancer reagent. Use LDV reservoirs rather than standard reservoirs for the highlighted reagents – the reservoir type must be manually adjusted for these reagents in the firefly protocol, and the reservoir starting volume calculation adjusted from using a 200µL dead volume to a 35µL dead volume. The overfill volume for the Ligation Master Mix + Enhancer must also be manually adjusted to 20µL in the firefly protocol.

Protocol overview

Protocol names reflect the section names and numbering used in the NEB instruction manual for the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (E7805/E6177, v4.0 7/23).

Protocol 1 of 4

1.1-1.2 Fragmentation, End Prep and Adaptor Ligation

Input DNA is transferred to a clean PCR plate using the pipetting head. NEBNext Ultra II FS Reaction Buffer and FS Enzyme Mix are added to the input DNA using the firefly dispense head, with the plate on a chilled thermal block. Reagents are mixed on the shaker.

The user moves the plate to the thermocycler to run the Fragmentation/End Prep program, then moves the plate back to the firefly deck on completion of the program.

NEBNext Ultra II Ligation Master Mix, Ligation Enhancer and Adaptor for Illumina are added to the plate using the firefly dispense head. Reagents are mixed on the shaker.

The user moves the plate to the thermocycler to run the Adaptor Ligation program. This is a safe stopping point where samples can be stored at -20°C if required. See Figure 2.

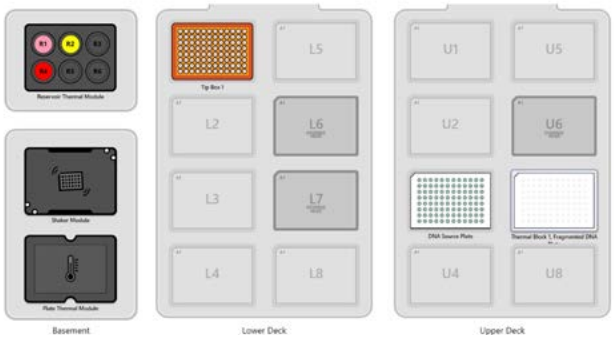

Figure 2. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 1 of 4: 1.1-1.2 Fragmentation, End Prep and Adaptor Ligation. Lower deck: (L1) Tip box 1 – 50 µL pipetting head tips; upper deck: (U3) DNA Source Plate, (U7) empty PCR plate on a thermal block; dispense head reservoirs: (R1) Adaptor - diluted as required, (R2) FS Enzyme + Buffer Mix, (R4) Ligation Master Mix + Enhancer. Example shown for 12 columns of samples.

Protocol 2 of 4

1.3 Adaptor Clean-up

When the Adaptor Ligation program on the thermocycler is complete, the user returns the plate to the firefly deck. The “Fragmented DNA Plate” now contains the ligation reaction product.

The ligation reaction product undergoes a 0.8X bead clean-up, where beads, 80% ethanol and elution buffer are dispensed using the firefly dispense head, and fresh pipetting head tips are used for mixing the beads with the sample, and for all ethanol and supernatant removals. The cleaned-up adaptor ligated libraries are transferred to a fresh plate. This is a safe stopping point where samples can be stored at -20°C if required. See Figure 3 for the starting deck layout for this protocol.

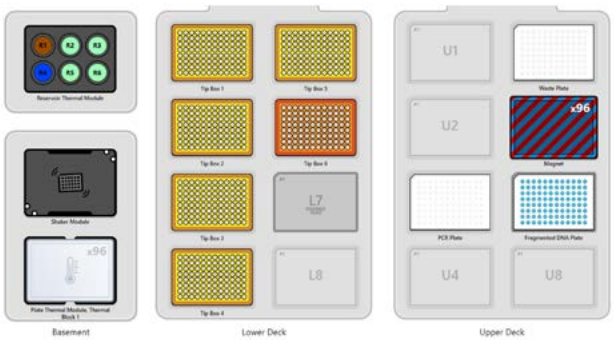

Figure 3. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 2 of 4: 1.3 Adaptor Clean-up. Lower deck: (L1-L5) Tip box 1-5 – 125 µL pipetting head tips, (L6) Tip box 6 – 50 µL pipetting head tips; upper deck: (U3) empty PCR Plate, (U5) Waste Plate, (U6) Alpaqua Magnum FLX magnet, (U7) Fragmented DNA plate - containing fragmented, adaptor-ligated DNA; dispense head reservoirs: (R1) Beads, (R4) 0.1X TE, (R2, R3, R5, R6) 80% Ethanol. Example shown for 12 columns of samples.

Protocol 3 of 4

1.4 PCR Enrichment

This step deviates from the standard manual protocol. Here, USER is added to the PCR reaction mix and the USER and PCR incubations are combined (see Table 4).

NEBNext Ultra II Q5 Master Mix, USER enzyme and Unique Dual Index Primer Pairs are added to the adaptor-ligated libraries and are mixed on the shaker.

IMPORTANT: In advance of protocol initiation, the seal on primer index plate must be pierced to permit unobstructed access to the wells being used.

On completion of this protocol, the plate is moved to the thermocycler and the USER-PCR program should be run with an appropriate number of cycles – see Table 4 for the cycling conditions. Figure 4 shows the starting deck layout for this protocol.

Step name	Temperature (°C)	Time	Cycles
USER incubation	37°C	15 minutes	1
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	4-9*
Annealing/ Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	forever	

Table 4. Example of the cycling conditions required for the USER-PCR thermocycler program

**PCR cycle numbers for 1-200ng input used to test this workflow on firefly. Please refer to the NEBNext Ultra II FS DNA manual for cycle number recommendations for other input amounts.*

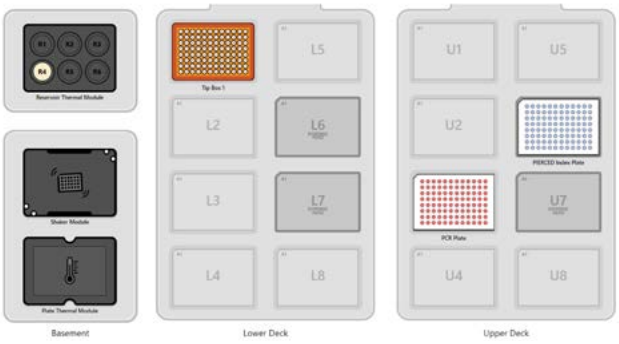

Figure 4. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 3 of 4: 1.4 PCR Enrichment. Lower deck: (L1) Tip box 1 – 50 µL pipetting head tips; upper deck: (U3) PCR Plate, (U6) Index Plate with pre-pierced wells; dispense head reservoirs: (R4) NEBNext Ultra II Q5 Master Mix + USER enzyme. Example shown for 12 columns of samples.

Protocol 4 of 4

1.5 PCR Clean-up

When the USER-PCR program on the thermocycler is complete the user returns the plate to the firefly deck.

A 0.9X bead clean-up is performed on the PCR product, where beads, 80% ethanol and elution buffer are dispensed using the firefly dispense head, and fresh pipetting head tips are used for mixing the beads with the sample, and for all ethanol and supernatant removals. The cleaned-up libraries are moved to a fresh plate. See Figure 5 for the starting deck layout for this protocol.

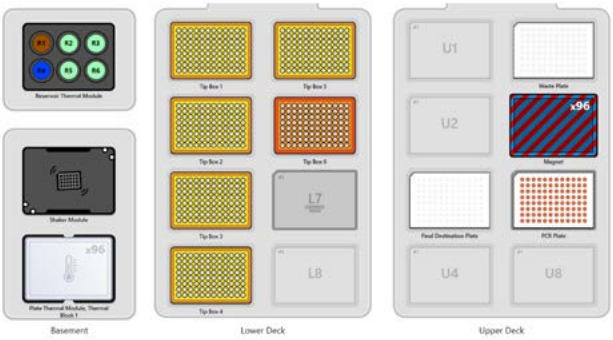

Figure 5. Starting deck layout for firefly NEBNext Ultra II FS DNA Protocol 4 of 4: 1.5 PCR Clean-up. Lower deck: (L1-L5) Tip box 1-5 – 125 µL pipetting head tips, (L6) Tip box 6 – 50 µL pipetting head tips; upper deck: (U3) empty Final Destination Plate, (U5) Waste Plate, (U6) Alpaqua Magnum FLX magnet, (U7) PCR Plate; dispense head reservoirs: (R1) Beads, (R4) 0.1X TE, (R2, R3, R5, R6) 80% Ethanol. Example shown for 12 columns of samples.

Optional protocol

2.3 Size Selection for DNA Input ≥ 100 ng

This optional Size Selection protocol can be run instead of protocol 2 of 4 (1.3 Adaptor Clean-up). See Figure 6 for the starting deck layout for this protocol.

The Fragmented DNA plate contains fragmented, adaptor-ligated DNA and starts with 68.5µL in each well. 33.5µL of 0.1X TE is added to each sample to give a final volume of 102µL. A double-sided size selection takes place where the volume of the 1st and 2nd bead addition is set as a variable so the user can modify these volumes as required.

A default bead volume of 40µL per well is set for the 1st bead addition. Following the bead addition, tip mixing and incubation on the magnet, the supernatant is transferred to a fresh plate (Intermediate Plate), leaving 4µL per well in the source plate.

A default bead volume of 20µL per well is set for the 2nd bead addition. Following the bead addition, tip mixing and incubation on the magnet, the supernatant is transferred to a fresh plate (PCR Plate), leaving 2µL per well in the source plate.

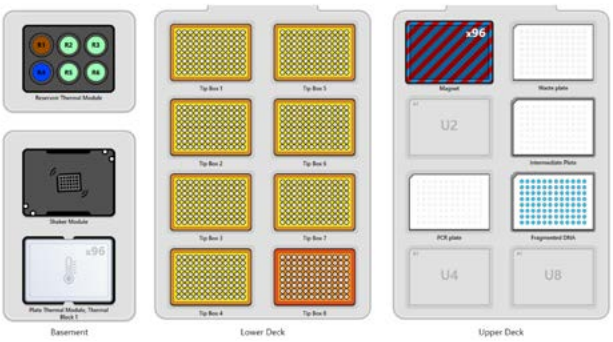

Figure 6. Starting deck layout for firefly NEBNext Ultra II FS DNA Optional protocol: 12.3 Size Selection of Adaptor-ligated DNA for DNA Input ≥ 100 ng. Lower deck: (L1-L7) Tip box 1-7 – 125 µL pipetting head tips, (L8) Tip box 8 – 50 µL pipetting head tips; upper deck: (U1) Alpaqua Magnum FLX magnet, (U3) empty PCR Plate, (U5) Waste Plate, (U6) empty Intermediate Plate, (U7) Fragmented DNA plate - containing fragmented, adaptor-ligated DNA; dispense head reservoirs: (R1) Beads, (R4) 0.1X TE, (R2, R3, R5, R6) 80% Ethanol. Example shown for 12 columns of samples.

Parts required

Supplier	Part	Part number	Number required
NEB	NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina	E7805 or E6177 (with beads)	1
NEB	NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs) Sets 1-5	E6440, E6442, E6444, E6446 and E6448	1
eppendorf	twin.tec® PCR Plate 96	30128648	4*
Greiner	Microplate 96 well F-bottom (waste plate)	655901	1
SPT Labtech	EZ-Load Pipette Tips, 100 µL, with Filters, Sterile, 96 Tips per Rack, 40 Racks per Case	125-096-FF-FS	10† boxes
SPT Labtech	EZ-Load Pipette Tips, 35 µL, with Filters, Sterile, 96 Tips per Rack, 40 Racks per Case	050-096-FF-FS	4 boxes
SPT Labtech	dragonfly® discovery syringes (pack 100 syringes / plungers	4150-07200	16 syringes
SPT Labtech	dragonfly® reservoirs (pack/50)	4150-07103	14 reservoirs
SPT Labtech	dragonfly® discovery low dead volume reservoirs (pack 25)	4150-07202	2 reservoirs
Alpaqua Engineering	Alpaqua Magnum FLX (96 well ring magnet)	A000400	1
SPT Labtech	firefly Thermo block 96	3276-01065	1

Table 5. Parts required to process 96 samples through the NEBNext Ultra II FS DNA Library Prep protocols 1 to 4 (DNA input <100 ng) on firefly with no reuse of tips, syringes or reservoirs between protocols. If DNA input ≥100 ng and the size selection (optional protocol) is used, amend the number of parts required to *5 and †12.