*FUSION*Plex™ for Illumina®—Workflow Overview

Follow the instructions and incubations for each step. All mixing steps should be performed on ice. Pipette up and down 8 times or vortex to mix after re-suspending each lyosphere and spin down prior to incubations and transfers. For incubations, use a lid heated ≥100°C except where specified otherwise.

Random Priming

Add 20-250 ng RNA to Random Priming reagent for a total volume of 20 µL.

Incubate as indicated.

Step	Incubation temperature	Incubation time
1	65°C	5 min
2	4°C	Hold

First Strand cDNA Synthesis

Transfer 20 µL Random Priming mixture to First Strand cDNA Synthesis reagent.

Incubate as indicated.

Transfer 1 µL First Strand cDNA Synthesis mixture to 9 µL water for PreSeq RNA QC Assay.

Step	Incubation temperature	Incubation time
1	25°C	10 min
2	42°C	30 min
3	80°C	20 min
4	4°C	Hold

Second Strand cDNA Synthesis

Add 21 µL ultrapure water to 19 µL First Strand cDNA Synthesis mixture and then transfer 40 µL to Second Strand cDNA Synthesis reagent.

Incubate as indicated.

Optional stopping point after this step. Store at –10°C to –30°C.

Step	Incubation temperature	Incubation time
1	16°C	60 min
2	75°C	20 min
3	4°C	Hold

PreSeq RNA QC Assay

Build qPCR reaction and incubate as per your master mix-specific instructions.

iTaq SYBR Green Supermix (Bio-Rad) – 5 µL
10X VCP Primer Mix – 1 µL
Diluted cDNA sample or NTC – 4 µL

Incubate as indicated.

Incubation temperature	Incubation time	# of cycles
95°C	20 [20*] sec	1
95°C	3 [15*] sec	35
60°C	30 [60*] sec	
60–95°C	0.5°C/sec increment	1
* Times in [ ] are for standard cycling		

End Repair

Transfer 40 µL Second Strand cDNA Synthesis mixture to End Repair reagent.

Incubate as indicated with unheated lid.

Step	Incubation temperature	Incubation time
1	25°C	30 min
2	4°C	Hold
AMPure™ XP clean-up (100 µL). Elute in 20 µL.		

Ligation Step 1

Transfer 20 µL End Repair mixture to Ligation Step 1 reagent.

Incubate as indicated.

After incubation add 20 µL of 10 mM Tris-HCl, pH 8.0 and mix.

Step	Incubation temperature	Incubation time
1	37°C	15 min
2	4°C	Hold
AMPure XP clean-up (50 µL). Elute in 42 µL.		

MBC Adapters

Transfer 40 µL Ligation Step 1 mixture to the MBC adapters.

Ligation Step 2

Transfer the entire volume of the MBC adapters to Ligation Step 2 reagent.

Incubate as indicated with unheated lid.

Optional stopping point before purification. Store at -10°C to -30°C.

Step	Incubation temperature	Incubation time
1	22°C	5 min
2	4°C	Hold
Ligation clean-up beads (50 µL). Elute in 18 µL 5 mM NaOH, 75°C 10 min.		

First PCR

Add 2 µL GSP1 primers to the First PCR HS reagent.
Then add 18 µL of purified Adapter Ligation mixture and mix.

Incubate as indicated.

Optional stopping point after this step.
Store at –10°C to –30°C.

Incubation temperature	Incubation time	# of cycles
95°C	3 min	1
95°C	30 sec	Varies*
Varies*	10 sec	
Varies*	Varies*	
72°C	3 min	1
4°C	Hold	1
AMPure XP clean-up (24 µL). Elute in 20 µL.		

Second PCR

Add 2 µL GSP2 primers to Second PCR reagent.
Then add 18 µL of purified First PCR mixture and mix.

Incubate as indicated.

Optional stopping point after this step.
Store at –10°C to –30°C.

Incubation temperature	Incubation time	# of cycles
95°C	3 min	1
95°C	30 sec	Varies*
Varies*	10 sec	
Varies*	Varies*	
72°C	3 min	1
4°C	Hold	1
AMPure XP clean-up (24 µL). Elute in 20 µL.		

Proceed with Protocol: Quantify, Normalize, and Sequence Protocol for Illumina®

Appendix: AMPure® XP bead purification†

1. Add AMPure XP beads to reaction
2. Mix to homogeneous solution
3. Incubate at RT for 5 min
4. Briefly spin down
5. Place on magnet for 4 min
6. Discard supernatant carefully
7. Wash beads 2x with 200 µL fresh 70% EtOH
8. Remove residual fluid with 20 µL pipette
9. Air dry 3–5 min at RT
10. Elute DNA with volume* of 10 mM Tris-HCl pH 8.0
11. Place on magnet for 2 min
12. Remove purified product

* Refer to Product Insert for panel-specific parameters

† Work at room temperature for bead purifications

Appendix: Ligation cleanup bead purification†

1. Perform buffer exchange with 50 µL of fresh Ligation cleanup buffer
2. Combine 50 µL Ligation cleanup beads with entire volume Ligation Step 2 reaction
3. Mix solution by vortexing and incubate 5 min (2x)
4. Briefly spin down
5. Place tubes on magnet for 1 min
6. Discard supernatant carefully
7. Wash beads 2x with 200 µL Ligation cleanup buffer
8. Wash beads with 200 µL ultrapure H_2O
9. Resuspend beads in volume* 5 mM NaOH
10. Incubate reactions 10 min at 75°C
11. Cool reactions to 4°C
12. Briefly spin down
13. Place on magnet for 2 min
14. Remove purified product

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