

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  $\geq 100^{\circ}$ C, except where specified otherwise.

Priming   Incubate as indicated ←   2   4°C   He     2   4°C   He   2   4°C   He     2   4°C   He   2   4°C   He     First Strand cDNA Synthesis   Transfer 20 μL Random Priming mixture to First Strand cDNA Synthesis reagent.   Step   Incubation   Incubation     1   25°C   10   1   25°C   10   1     2   42°C   30   3   80°C   20   1     3   80°C   20   4   4°C   He	min old me min min min old
First Strand cDNA SynthesisTransfer 20 $\mu$ L Random Priming mixture to First Strand cDNA Synthesis reagent.StepIncubation TemperatureIncubation Time Time Time Time Time To 9 $\mu$ L water for PreSeq RNA QC Assay.24°CHo	min min min min old
Strand cDNA Synthesis reagent.Strand cDNA Synthesis reagent.TemperatureTimeIncubate as indicated cDNA SynthesisTransfer 1 μL First Strand cDNA Synthesis mixture to 9 μL water for PreSeq RNA QC Assay.380°C2044°CHorizontal	me min min min old
Strand cDNA Synthesis reagent.StepTemperatureTimeFirst Strand cDNA SynthesisTransfer 1 μL First Strand cDNA Synthesis mixture to 9 μL water for PreSeq RNA QC Assay.380°C2044°CHorizontal	me min min min old
First Strand cDNA SynthesisIncubate as indicated ✓242°C30Transfer 1 μL First Strand cDNA Synthesis mixture to 9 μL water for PreSeq RNA QC Assay.380°C2044°CHo	min min old
cDNA Synthesis $2$ $42^{\circ}$ C $30^{\circ}$ CTransfer 1 µL First Strand cDNA Synthesis mixture to 9 µL water for PreSeq RNA QC Assay. $3$ $80^{\circ}$ C $20^{\circ}$ C4 $4^{\circ}$ CHe	min
SynthesisTransfer 1 μL First Strand cDNA Synthesis mixture380°C20to 9 μL water for PreSeq RNA QC Assay.44°CHo	old
to 9 μL water for PreSeq RNA QC Assay.	
	ation
	ation
Step	me
Second Strand cDNA Synthesis reagent. 1 16°C 60	min
	min
	bld
Synthesis Optional stopping point after this step. Store at -10°C to -30°C.	
Build qPCR reaction and incubate as per your master mix-specific instructions.Incubation TemperatureIncubation Time	cycles
	1
iTaq SYBR Green Supermix (Bio-Rad) – 5 μL55 C20 [20 ] 3CCPreSeq10X VCP Primer Mix – 1 μL95°C3 [15#] sec	
Diluted cDNA sample or NTC – 4 μL3RNA QC60°C30 [60#] sec	35
Assay Incubate as indicated - 60-95°C 0.5°C/sec increment	1
# Times in [] are for standard cycling	
Indisiel 40 µE second strand conversion strand conversion strand	oation me
1 25°C 30	min
End Incubate as indicated with <u>unheated lid</u> 4°C Ho	bld
Repair AMPure <sup>™</sup> XP clean-up (100 μL). Elute in	n 20µL

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	Transfer 20 μL End Repair mixture to Ligation Step 1 reagent.	Step	Incubation Temperature	Incubation Time
Ligation Step 1	Incubate as indicated 👉	1	37°C	15 min
		2	4°C	Hold
	AMPure <sup>™</sup> XP clean-up (50 μL). Elute in 42 μl			
МВС	Transfer 40 $\mu L$ Ligation Step 1 mixture to the MBC ac	lapters.		
Adapters	Transfer the entire volume of the MBC adapters to Ligation Step 2 reagent.	Step	Incubation Temperature	Incubation Time
	Incubate as indicated with <u>unheated lid</u>	1	22°C	5 min
Ligation	incubate as indicated with <u>unneated ind</u>	2	4°C	Hold
Step 2	<b>STOP</b> Optional stopping point before purification. Store at -10°C to -30°C.		n-up beads (50 μL). nM NaOH, 75°C 10	
First PCR	Add 2 $\mu$ L GSP1 primers to the First PCR reagent. Then add 18 $\mu$ L of purified Adapter Ligation mixture and mix.	Incubation Temperature	Incubation Time	# of cycles
		95°C	3 min	1
	Incubate as indicated 🗲	95°C	30 sec	
		65°C *	5 min* (ramp rate 100%)	15*
		72°C	3 min	1
	Optional stopping point after this	4°C	Hold	1
	step. Store at -10°C to -30°C.	AMPure <sup>TM</sup> XP clean-up (24 $\mu L$ ). Elute in 20 $\mu L$		
Second PCR	Add 2 $\mu L$ GSP2 primers to Second PCR reagent. Then add 18 $\mu L$ of purified First PCR mixture and mix.	Incubation Temperature	Incubation Time	# of cycles
		95°C	3 min	1
	Incubate as indicated 🗲	95°C	30 sec	
		65°C *	5 min* (ramp rate 100%)	20*
		72°C	3 min	1
		4°C	Hold	1
	STOP Optional stopping point after this step. Store at -10°C to -30°C.	AMPure <sup>™</sup> X	P clean-up (24 μL).	Elute in 20 µL
		er to Product In	sert for panel-speci	fic parameters
	Proceed with Ion Torrent <sup>™</sup> Library Quantification Assay and Sequence			

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