SureSeq™

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SureSeq Quick Reference Guide – Good Quality DNA

This guide is intended as a quick reference for the SureSeq Library Preparation Kit protocol. Please reference the full version of the handbook for details or laboratory specific guidelines.

Note that tables have been coloured to match the lid colour of the Library Preparation Kit reagent being used.

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DNA Fragmentation

Shear DNA using Covaris or Fragmentase

DNA: Calculate total ng of DNA input needed for specific panel.

Covaris: Expected size 150-200 bp

Sample Type	Total Sample Volume (µl)	Shearing Time
Good quality DNA >200 ng	130	6 cycles, 1 min each
Good quality DNA <200 ng	50	180 secs

1. Prepare DNA in TE to the final volume listed above.
2. Transfer to Covaris tube
3. Shear using appropriate time.

Fragmentase: Expected size 150-250 bp

NB Prepare and store all reagents on ice

Reagent	Volume (µl)
10x Fragmentase Reaction Buffer v2	2
dsDNA Fragmentase Enzyme	2
Total	**4**

1. Prepare DNA in nuclease free water to a total volume of 16 µl.
2. Make master mix with the Enzyme Buffer and Enzyme. Ensure the enzyme is mixed well prior to use.
3. Add 4 µl to each DNA tube
4. Incubate at 37°C for 30 min
5. Immediately add 5 µl of 0.5 M EDTA and pipette mix
6. Proceed to post- fragmentation purification

Post-Fragmentation Purification

Use room temperature AMPure XP beads and freshly prepared 70% ethanol.

1. Add appropriate volume of AMPure XP beads to each fragmented sample

Fragmentation Method	Starting amount of DNA (ng)	Volume beads (µl)
Covaris	>200	180
	<200	70
Fragmentase		45

2. Incubate for 5 minutes
3. Place on magnetic stand for approximately 3-5 min or until solution is clear.
4. Remove and discard supernatant
5. Add 500 µl or 200 µl of 70% ETOH, incubate 1 min, remove ETOH
6. Repeat step 5.

7. Dry beads/sample at 37°C for 1 min or until the residual ethanol evaporates and the bead pellet is matte.
8. Add 27 µl nuclease-free water to bead pellet, mix and incubate for 5 mins
9. Place on magnetic tube for 2-3 mins or until solution is clear.
10. Remove 25 µl supernatant and transfer to fresh tube or plate.

End Repair and 3' End 'A' Tailing

Prepare Master Mix

Prepare a master mix on ice. Use reagents with green cap.

1. Prepare the master mix on ice as shown:

Reagent	Volume (µl)/ library	8 x library (µl)	16 x library (µl)
Nuclease-free H_2O	9	81	153
Step 1: ER Buffer	10	90	170
Step 2: ER Enzyme Mix	6	54	102
TOTAL	**25**	**225**	**425**

2. Add 25 µl master mix to 25 µl fragmentated DNA and mix
3. Run thermocycler as follows:

Step	Temperature (°C)	Time
1	20	30 min
2	72	30 min
3	4	Hold

IMPORTANT: Ensure samples are held at 4°C for 5-10 min, then proceed immediately with adaptor ligation.

Adaptor Ligation

Prepare Master Mix

1. Prepare a master mix on ice using the volumes shown depending on initial DNA concentration input. Use reagents with yellow cap.

If starting DNA is 1-3 µg	Volume (µl)/ library	8 x library (µl)	16 x library (µl)
Step 2: Ligation Buffer	3	27	51
Step 2: Adaptor A	5	45	85
Step 2: Adaptor B	5	45	85
Step 2: Ligase	2	18	34
Nuclease-free H_2O	0	-	-
TOTAL	**15**	**135**	**255**

If starting DNA is 0.5- 0.99 µg	Volume (µl)/ library	8 x library (µl)	16 x library (µl)
Step 2: Ligation Buffer	3	27	51
Step 2: Adaptor A	1.5	13.5	25.5
Step 2: Adaptor B	1.5	13.5	25.5
Step 2: Ligase	2	18	34
Nuclease-free H_2O	7	63	119
TOTAL	**15**	**135**	**255**

If starting DNA is 0.1- 0.25 µg	Volume (µl)/ library	8 x library (µl)	16 x library (µl)
Step 2: Ligation Buffer	3	27	51
Step 2: Adaptor A	0.5	4.5	8.5
Step 2: Adaptor B	0.5	4.5	8.5
Step 2: Ligase	2	18	34
Nuclease-free H_2O	9	81	153
TOTAL	**15**	**135**	**255**

If starting DNA is <0.1 µg	Volume (µl)/ library	8 x library (µl)	16 x library (µl)
Step 2: Ligation Buffer	3	27	51
Step 2: Adaptor A	0.25	2.25	4.25
Step 2: Adaptor B	0.25	2.25	4.25
Step 2: Ligase	2	18	34
Nuclease-free H_2O	9.5	85.5	161.5
TOTAL	**15**	**135**	**255**

2. Add 15 µl of the reaction mix to each well or tube on ice.
3. Incubate in a thermal cycler without a heated lid as follows:

Temperature (°C)	Time
20	15 min

4. Proceed immediately to Ligated Library Purification.

Ligated Library Purification

Use room temperature AMPure XP beads and freshly prepared 70% ETOH.

1. Add 117 µl of AMPure XP beads to each sample
2. Incubate for 5 minutes
3. Place on magnetic stand for approximately 3-5 min or until solution is clear.
4. Remove and discard supernatant
5. Add 200 µl 70% ETOH, incubate 1 min, remove ETOH
6. Repeat step 5.
7. Dry beads/sample at 37°C for 1 min or until bead pellet is matte
8. Add 32 µl nuclease-free water to bead pellet, mix and incubate for 5 mins
9. Place on magnetic tube for 2-3 mins or until solution is clear.

10. Remove 30 µl supernatant and transfer to fresh tube or plate.

Ligated Library Quality Control

Use Qubit High Sensitivity (HS) kit.

Expected concentration values:

Shearing method	Starting DNA (ng)	Library (ng/µl)
Covaris	1000	15-20
	500	5-15
	200	3-5
	100	<3
Fragmentase	1000	7-15
	500	5-7
	200	1.5-5
	100	<1.5

STOPPING POINT: Store samples at 4°C if not used immediately.

PCR 1

Determine number of PCR cycles:

Use the number of cycles for PCR 1 based on the type of starting material and concentration of ligated library.

Ligated Library Concentration (ng)	Cycle numbers	Expected Sequencing Duplication
>9 ng	6	≤10%
2–8 ng	8	10 – 30%
1–1.9 ng	10	30 – 40%
>1 ng	12 Always use 30 µl of ligation product	40+%

Prepare Master Mix

1. Prepare the master mix on ice using orange capped reagents:

For libraries made with >200ng, use 15 µl of Ligated Library	*1x library (µl)*	*8x library (µl)*	*16x library (µl)*
Nuclease-free H_2O	23	207	391
Step 3: PCR Buffer	5	45	85
Step 3: Primer Mix	5	45	85
Step 3: DNA Polymerase	2	18	34
TOTAL	**35**	**315**	**595**

For libraries made with < 200ng, use 30 µl of Ligated Library	*1x library (µl)*	*8x library (µl)*	*16x library (µl)*
Nuclease-free H_2O	8	72	136
Step 3: PCR Buffer	5	45	85
Step 3: Primer Mix	5	45	85
Step 3: DNA Polymerase	2	18	34
TOTAL	**20**	**180**	**340**

2. Add appropriate volume of the master mix, 35 µl or 20 µl, to each sample.
3. Run thermocycler as follows with predetermined cycle numbers.

Step	Temperature (°C)	Time
1	98	3 min
2	98	30 sec
3	65	30 sec
4	72	1 min
5		Repeat Step 2 to Step 4: Predetermined # of cycles
6	72	10 min
7	4	Hold

PCR-1 Purification

Use room temperature AMPure XP beads and freshly prepared 70% ETOH.

11. Add 90 µl of AMPure XP beads to each sample
12. Incubate for 5 minutes
13. Place on magnetic stand for approximately 3-5 min or until solution is clear.
14. Remove and discard supernatant
15. Add 200 µl 70% ETOH, incubate 1 min, remove ETOH
16. Repeat step 5.
17. Dry beads/sample at 37°C for 1 min or until bead pellet is matte
18. Add 32 µl nuclease-free water to bead pellet, mix and incubate for 5 mins
19. Place on magnetic tube for 2-3 mins or until solution is clear.
20. Remove 30 µl supernatant and transfer to fresh tube or plate.

PCR-1 Quality Control

Use Qubit Broad Range (BR) and DNA1000 Tapestation***** to assess quality

	Expected Value
D1000 Tapestation	180 to 275 bp (+/- 10%)
BR Qubit	40 to 80 ng/µl

STOPPING POINT: Store samples at -20°C if not used immediately

***Note:** This is an optional QC

Hybridisation

Prepare Libraries for hybridisation

The hybridisation protocol may vary according to the panel. Verify amounts and times prior to performing.

1. Aliquot 1000 or 500 ng of each prepared library into 0.2 ml tubes.
2. Prepare pre-hybridsation master mix as shown

Reagent	1x library (µl)	8x library (µl)	16x library (µl)
COT Human DNA (1mg/ml)	5	45	85
OGT Blocking Oligos	0.6	5.4	10.2
Total	**5.6**	**50.4**	**95.2**

3. Add 5.6 µl master mix to each sample.
4. Dry down tubes/plate using SpeedVac or similar evaporation device.
5. Resuspend in 2.5 µl of nuclease-free water.

NOTE: For optimal recovery, incubate the sample at 37°C for 20 min and mix occasionally on a vortex or store at 4°C overnight.

Hybridisation Mix

Prepare the hybridisation mix at room temperature.

1. Prepare master mix as shown

Reagent	1x library (µl)	8x library (µl)	16x library (µl)
Vial 5: NimbleGen 2x Hybridization Buffer	7.5	67.5	127.5
Vial 6: NimbleGen Hybridization Component A	3	27	51
SureSeq panel baits	2	18	34
Total	**12.5**	**112.5**	**212.5**

2. Add 12.5 µl to each sample.
3. Run thermocycler as follows with heated lid (105°C):

Step	Temperature (°C)	Time
1	95	5 min
2	65	∞

a. Incubate mixture at 65°C for the recommended times for specific panel used.
NOTE: Panel type may change incubation times.
b. General guideline as follows based on starting material:

Panel	Incubation Time
Ovarian, Myeloid and all MyPanels	4 hours
CoreMPN	30 mins

Prepare Capture and Buffers

Prepare another thermocycler next to hybridisation for best results. Set hybridisation thermocycler to hold at either 35°C or 40°C. Prepare buffers and pre-warm prior to hybridization washes for a minimum of 30 mins before use. Aliquot appropriate amounts into 0.2 ml strip tubes or plates for ease of use.

1. Dilute the 10x wash buffers (I, II, III and Stringent) and the 2.5x bead wash buffer to 1x working solutions as shown:

Volumes per sample:

Reagent	Buffer (µl)	Water (µl)	1X Final Volume (µl)
10x Wash Buffer I	30	270	300
10x Wash Buffer II	20	180	200
10x Wash Buffer III	20	180	200
10x Stringent Wash Buffer	40	360	400
2.5x Bead Wash Buffer	200	300	500

Volumes for 8 samples:

Reagent	Buffer (µl)	Water (µl)	1X Final Volume (µl)
10x Wash Buffer I	270	2430	2700
10x Wash Buffer II	180	1638	1818
10x Wash Buffer III	180	1638	1818
10x Stringent Wash Buffer	360	3258	3618
2.5x Bead Wash Buffer	1800	2700	4500

Volume for 16 samples:

Reagent	Buffer (µl)	Water (µl)	1X Final Volume (µl)
10x Wash Buffer I	510	4590	5100
10x Wash Buffer II	340	3060	3400
10x Wash Buffer III	340	3060	3400
10x Stringent Wash Buffer	680	6120	6800
2.5x Bead Wash Buffer	3400	5100	8500

2. Aliquot and pre-warm the following wash buffers to 65°C:

Reagent	Volume (µl)/ Sample
1X Stringent Wash Buffer	200
1X Stringent Wash Buffer	200
1X Wash Buffer I	100

3. Aliquot and pre-warm the following wash buffers to 35°C:

Reagent	Volume (µl)/ Sample
1X Wash Buffer I	200
1X Wash Buffer II	200
1X Wash Buffer III	200

Prepare Streptavidin Beads

Allow the M270 Streptavidin Magnetic Beads to equilibrate to room temperature for 30 mins before use.

1. Add 100 µl M270 magnetic beads per sample (combine up to 400 µl of beads per 1.5 ml microfµge tube).
2. Place on magnetic stand for approximately 1 min or until solution is clear.
3. Remove and discard supernatant.
4. Add 200 µl of 1X Bead Wash Buffer per 100 µl beads. Vortex mix and place on magnetic rack.
5. Remove and discard supernatant.
6. Repeat step 4-5.
7. Resuspend beads in 100 µl per sample of 1X Bead Wash Buffer (If 400 µl beads used, resuspend in 400 µl 1X Bead Wash Buffer).
8. Transfer 100 µl of beads into new tube/plate for capture reaction.
9. Place tube/plate on magnetic stand until solution is clear. Remove and discard supernatant.
10. Proceed immediately to Hybrid Capture. Do not allow beads to dry.

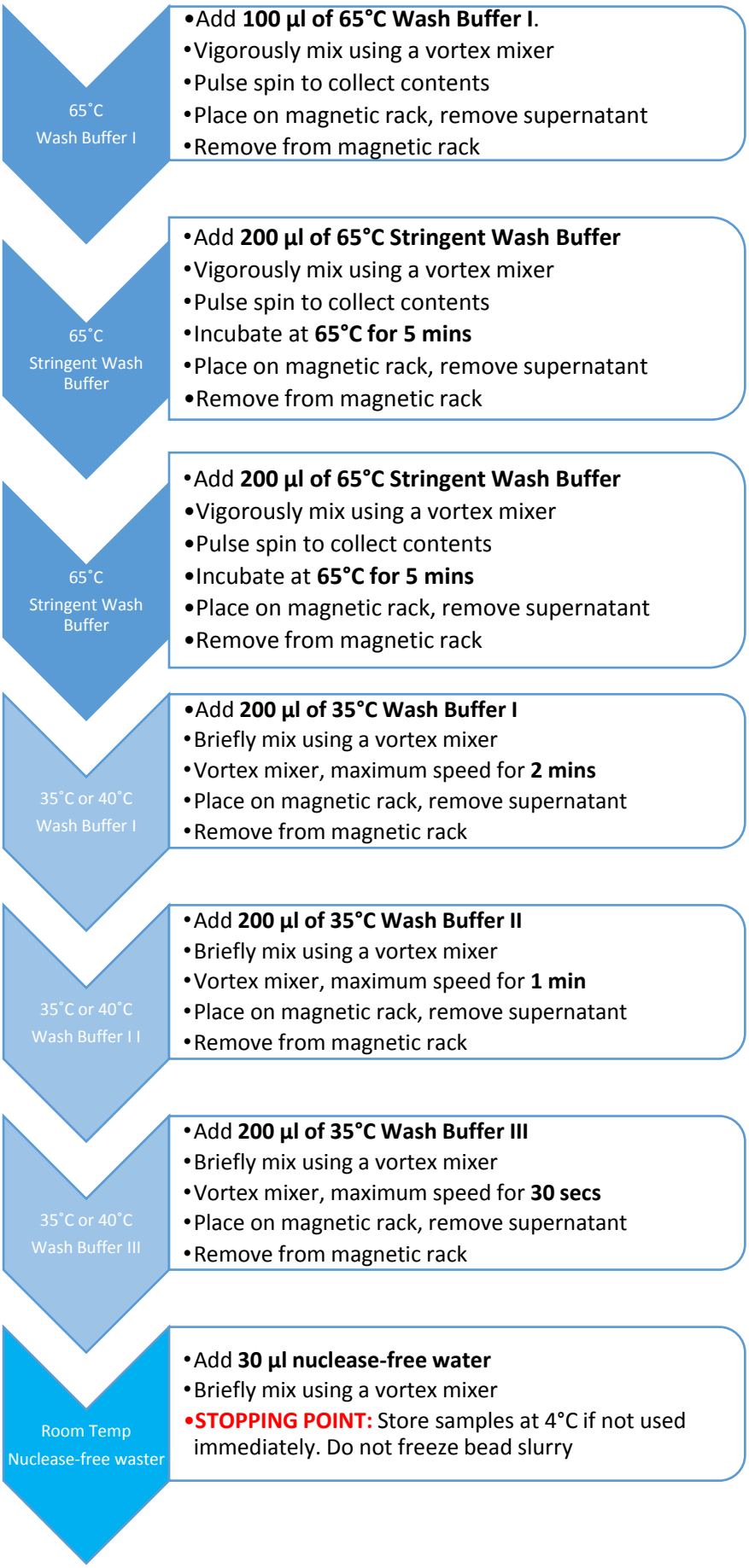
Hybrid Capture

1. After hybridisation incubation, transfer 15 µl of hybridized sample to prepared streptavidin beads.
2. Vortex to mix to ensure beads are resuspended.
3. Place samples back on thermocycler at 65°C for 45 min (Or 15 min if using Core MPN panel)
4. Ensure that the heated lid is on (set at 105°C)
5. Mix for 3 secs every 15 mins (or 5 min if using Core MPN panel)
6. Briefly spin tubes.
7. Proceed immediately to Wash Samples

Wash Streptavidin Beads to remove unbound DNA

Temp °C	*Reagent*	*Directions*
65	Wash Buffer I	1. Add **100 µl of 65°C Wash Buffer I**. 2. Vigorously mix using a vortex mixer 3. Pulse spin to collect contents 4. Place sample on magnetic rack, discard **115 µl of buffer/hyb mix** 5. Remove tubes from rack
	Stringent Wash Buffer	6. Add **200 µl of 65°C Stringent Wash Buffer** 7. Vigorously mix using a vortex mixer 8. Pulse spin to collect contents 9. Incubate at **65°C for 5 mins** 10. Place on magnetic rack, remove supernatant 11. Remove from magnetic rack
	Stringent Wash Buffer	12. Add **200 µl of 65°C Stringent Wash Buffer** 13. Vigorously mix using a vortex mixer 14. Pulse spin to collect contents 15. Incubate at **65°C for 5 mins** 16. Place on magnetic rack, remove supernatant 17. Remove from magnetic rack
35	Wash Buffer I	18. Add **200 µl of 35°C Wash Buffer I** 19. Briefly mix using a vortex mixer 20. Vortex mixer, maximum speed for **2 mins** 21. Place on magnetic rack, remove supernatant 22. Remove from magnetic rack
	Wash Buffer II	23. Add **200 µl of 35°C Wash Buffer II** 24. Briefly mix using a vortex mixer 25. Vortex mixer, maximum speed for **1 min** 26. Place on magnetic rack, remove supernatant 27. Remove from magnetic rack
	Wash Buffer III	28. Add **200 µl of 35°C Wash Buffer III** 29. Briefly mix using a vortex mixer 30. Vortex mixer, maximum speed for **30 secs** 31. Place on magnetic rack, remove supernatant 32. Remove from magnetic rack
Room Temp	Nuclease free Water	33. Add **30 µl nuclease-free water** 34. Briefly mix using a vortex mixer

STOPPING POINT: Store samples at 4°C if not used immediately. Do not freeze bead slurry.


65˚C
Wash Buffer I
•Add 100 µl of 65°C Wash Buffer I.
•Vigorously mix using a vortex mixer
•Pulse spin to collect contents
•Place on magnetic rack, remove supernatant
•Remove from magnetic rack
65˚C
Stringent Wash Buffer
•Add 200 µl of 65°C Stringent Wash Buffer
•Vigorously mix using a vortex mixer
•Pulse spin to collect contents
•Incubate at 65°C for 5 mins
•Place on magnetic rack, remove supernatant
•Remove from magnetic rack
65˚C
Stringent Wash Buffer
•Add 200 µl of 65°C Stringent Wash Buffer
•Vigorously mix using a vortex mixer
•Pulse spin to collect contents
•Incubate at 65°C for 5 mins
•Place on magnetic rack, remove supernatant
•Remove from magnetic rack
35˚C or 40˚C
Wash Buffer I
•Add 200 µl of 35°C Wash Buffer I
•Briefly mix using a vortex mixer
•Vortex mixer, maximum speed for 2 mins
•Place on magnetic rack, remove supernatant
•Remove from magnetic rack
35˚C or 40˚C
Wash Buffer I I
•Add 200 µl of 35°C Wash Buffer II
•Briefly mix using a vortex mixer
•Vortex mixer, maximum speed for 1 min
•Place on magnetic rack, remove supernatant
•Remove from magnetic rack
35˚C or 40˚C
Wash Buffer III
•Add 200 µl of 35°C Wash Buffer III
•Briefly mix using a vortex mixer
•Vortex mixer, maximum speed for 30 secs
•Place on magnetic rack, remove supernatant
•Remove from magnetic rack
Room Temp
Nuclease-free waster
•Add 30 µl nuclease-free water
•Briefly mix using a vortex mixer
•STOPPING POINT: Store samples at 4°C if not used immediately. Do not freeze bead slurry

Post-Capture PCR

Prepare Master Mix

Prepare samples on ice. Use reagents with purple cap.

4. Prepare the master mix as shown:

Reagent	1x library (µl)	8x library (µl)	16x library (µl)
Nuclease-free H_2O	23	207	391
Step 4: PCR Buffer	5	45	85
Step 4: Primer	1	9	17
Step 4: DNA Polymerase	2	18	34
TOTAL	**31**	**279**	**527**

5. Add 31 µl master mix to new tube/ plate.
6. Add 5 µl of sequencing index (1-48) to each well and mix. RECORD Index used for each sample.
7. Add 14 µl of Sample beads slurry to corresponding tube/ well.
8. Pipette mix and place on thermocycler with following conditions:

Step	Temperature (°C)	Time
1	98	3 min
2	98	30 sec
3	65	30 sec
4	72	1 min
5		Repeat Step 2 to Step 4 for 20
6	72	10 min
7	4	Hold

Post-Capture PCR Purification

Use room temperature AMPure XP beads and freshly prepared 70% ETOH.

1. Add 90 µl of AMPure XP beads to each sample
2. Incubate for 5 minutes
3. Place on magnetic stand for approximately 3-5 min or until solution is clear.
4. Remove and discard supernatant
5. Add 200 µl 70% ETOH, incubate 1 min, remove ETOH
6. Repeat step 5.
7. Dry beads/sample at 37°C for 1 min or until bead pellet is matte
8. Add 32 µl nuclease-free water to bead pellet, mix and incubate for 5 mins
9. Place on magnetic tube for 2-3 mins or until solution is clear.
10. Remove 30 µl supernatant and transfer to fresh tube or plate.

Post Capture PCR Quality Control

Use Qubit High Sensitivity (HS) and Tapestation to assess quality:

	Expected Value
Tapestation High Sensitivity	220-350 bp (+/- 10%)
HS Qubit	1 – 10 ng/µl for CoreMPN 5 - 15 ng/µl for Ovarian & Myeloid 10 – 20 ng/µl for MyPanel Myeloid 49 genes

NOTE: If the yield is <0.5 ng/µl repeat the PCR with the remaining bead slurry.

STOPPING POINT: If the samples are not to be used immediately, store them at 4°C overnight or at –20°C for long-term storage.

Sequencing

Prepare Sequencing Pool

Prepare the sequencing pool such that each index-tagged sample is present in equimolar amounts in the final pool. Reference appropriate sequencer pooling worksheet. The pool should be at 4 nM.

1. Complete the “Pool Parameter” and “Sample” tabs in the pooling worksheet.
2. Add appropriate volumes of each indexed library per “Volumes to pipette” tab to a new 1.5 mL LoBind tube.
3. Adjust final volume of the sequencing pool with nuclease free water to desired concentration of 4nM.
4. Use Qubit High Sensitivity (HS) and TapeStation High Sensitivity to assess quality
5. Complete the “Pool validation and dilution” tab to determine the molar concentration of the sequencing pool
6. Load sample for sequencing. Refer to appropriate **sequencer manufactures protocol.**

Legal information

NGS library prep assay

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