Quick Start Protocol

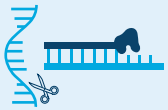
SureSelect XT HS2 RNA with Post-capture Pooling

G9989-90500 Rev A0

**For Research Use Only. Not for use in diagnostic procedures.

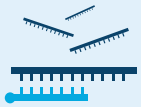
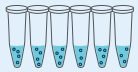
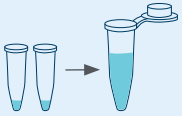
cDNA Conversion and Library Preparation Workflow

6 Hours

Fragment RNA and convert to cDNA	Clean-up cDNA	End repair and dA-tail cDNA	Ligate MBC-tagged adaptors	Clean-up ligation	Amplify and dual-index	Clean-up library	Quality control
							
2.5 hrs	30 min Stopping point	40 min	40 min	30 min	35 min	30 min	Varies Stopping point

Hybridization/Capture Workflow

4–5 Hours

Hybridize libraries to probe	Prepare streptavidin beads	Capture hybridized libraries	Amplify enriched libraries	Clean-up final libraries	Quality control	Pool libraries for multiplex NGS
						
1.5–2.5 hrs Optional overnight hold	30 min (Concurrent)	70 min	30 min	30 min	Varies Stopping point	30 min

Duration estimates are provided as guidelines for 16 reaction runs using 200 ng high-quality input RNA and probe design >5 Mb. Your results may vary.

This Quick Start Protocol provides key protocol details for experienced users. Visit SureSelect XT HS2 RNA System Protocol for the full User Guide with further information including:

- Safety information
- Notices to purchaser
- List of required materials
- FFPE sample qualification guidelines
- QC output examples
- Critical factors for system performance including component handling, mixing, and spinning specifications; pay special attention to the key critical factors such as mixing steps highlighted in this Quick Start Protocol
- Full protocol details including Notes and Caution statements
- Index sequences and other NGS support

RNA Fragmentation and Conversion to cDNA

Note: Reagent vial cap colors are indicated by colored circles adjacent to reagent use instructions.

Step 1: Add cDNA primers and fragment intact (non-FFPE) RNA samples

10 min

The 2X Priming Buffer includes both cDNA primers and heat-driven fragmentation agents. Intact (non-FFPE) RNA samples are fragmented by exposure to elevated temperature while FFPE RNA samples are kept on ice to prevent further fragmentation.

- [ ] If the run includes intact (non-FFPE) samples, set up thermal cycler program (Table 1). Start and pause program.

Table 1: Thermal cycler program for RNA fragmentation (vol 20 µl)

Step	Temperature	Time
Step 1	94 °C	4 min
Step 2	4 °C	1 min
Step 3	4 °C	Hold

- [ ] Prepare RNA samples in wells: 10 µl each containing 10–200 ng total RNA in nuclease-free water. Keep on ice.

Use ≥ 50 ng RNA for poor quality FFPE samples (DV200 of 20%–50%).

- [ ] ● Add 10 µl 2X Priming Buffer to all RNA samples. Mix **>** brief spin.
- [ ] Place FFPE RNA samples on ice. Place intact (non-FFPE) samples in thermal cycler and press play/continue.
- [ ] At 4 °C Hold step, remove fragmented non-FFPE samples from thermal cycler **>** keep on ice.

Step 2: Synthesize first-strand cDNA

55 min

- [ ] Set up thermal cycler program (Table 2). Start and pause program.

Table 2: Thermal cycler program for first-strand synthesis (vol 28 µl)

Step	Temperature	Time
Step 1	25 °C	10 min
Step 2	37 °C	40 min
Step 3	4 °C	Hold

- [ ] Vortex thawed First Strand Master Mix at high speed for 5 sec.

Reagent is viscous and must be vortexed.

- [ ] ● Add 8.5 µl First Strand Master Mix to RNA samples. Mix **>** brief spin.
- [ ] Place in thermal cycler and press play/continue.
- [ ] At 4 °C Hold step, remove from thermal cycler **>** keep on ice.

Note: Reagent vial cap colors are indicated by colored circles adjacent to reagent use instructions.

Step 3: Synthesize second-strand cDNA

65 min

- ☐ Set up thermal cycler program (Table 3). Start and pause program.

Table 3: Thermal cycler program for second-strand synthesis (vol 58 µl)

Step	Temperature	Time
Step 1	16 °C	60 min
Step 2	4 °C	Hold

- ☐ Vortex thawed Second Strand Enzyme Mix and Second Strand Oligo Mix at high speed for 5 sec.

Reagent is viscous and must be vortexed.

- ☐ ● Add 25 µl Second Strand Enzyme Mix to sample wells **>** keep on ice.
- ☐ ● Add 5 µl Second Strand Oligo Mix to sample wells **>** keep on ice.
- ☐ Mix>brief spin>place in thermal cycler and press play/continue.

Prep Ahead: Put AMPure XP beads at RT for use in next step (30 min equilibration)

Step 4: Clean-up cDNA

30 min

- ☐ Get AMPure XP beads held at RT, vortex until homogeneous.
- ☐ Add 105 µl of AMPure XP beads to each cDNA sample well. Mix **>** incubate at RT 5 min.
- ☐ Collect beads using magnet (2–5 min) **>** remove and discard supernatant.
- ☐ Wash beads 2X with 200 µl freshly-prepared 70% ethanol per wash.
  Washes completed: ☐ 1 ☐ 2
- ☐ After second wash removed, spin briefly **>** remove residual ethanol.
- ☐ Dry samples unsealed on the thermal cycler at 37 °C (1–2 min).
- ☐ Elute by adding 52 µl nuclease-free water to each sample well. Seal wells **>** vortex **>** brief spin. Incubate at RT 2 min.
- ☐ Collect beads using magnet (~5 min).
- ☐ Transfer 50 µl cleared supernatant to fresh well **>** keep on ice.

Stopping point

Library Preparation

Note: Reagent vial cap colors are indicated by colored circles adjacent to reagent use instructions.

Step 1: Prepare ligation master mix

5 min

- ☐ Vortex thawed Ligation Buffer at high speed for 15 sec.
  Reagent is viscous and must be vortexed.
- ☐ Prepare Ligation master mix (Lig MM, see Table 4). Mix **>** spin **> keep at RT** during end repair/A-tailing steps.

Table 4: Lig MM reagent volumes (including excess for supported run sizes)

Reagent		16 Rxn Kits		96 Rxn Kits	
	Per Rxn	8 Rxn	16 Rxn	24 Rxn	96 Rxn
Ligation Buffer	23 µl	207 µl	414 µl	598 µl	2300 µl
T4 DNA Ligase	2 µl	18 µl	36 µl	52 µl	200 µl
Total	25 µl	225 µl	450 µl	650 µl	2500 µl

Step 2: End repair and dA-tail fragments

40 min

- ☐ Set up thermal cycler program (Table 5). Start and pause program.

Table 5: Thermal cycler program for End repair/A-tailing (vol 70 µl)

Step	Temperature	Time
Step 1	20 °C	15 min
Step 2	72 °C	15 min
Step 3	4 °C	Hold

- ☐ Vortex thawed End Repair-A Tailing Buffer at high speed for 15 sec.
  Reagent is viscous and must be vortexed.
- ☐ Prepare End repair/A-tailing master mix (ER-AT MM, see Table 6). Mix **>** spin **>** keep on ice.

Table 6: ER-AT MM reagent volumes (including excess for supported run sizes)

Reagent		16 Rxn Kits		96 Rxn Kits	
	Per Rxn	8 Rxn	16 Rxn	24 Rxn	96 Rxn
End Repair-A Tailing Buffer	16 µl	144 µl	288 µl	416 µl	1600 µl
End Repair-A Tailing Enzyme Mix	4 µl	36 µl	72 µl	104 µl	400 µl
Total	20 µl	180 µl	360 µl	520 µl	2000 µl

- ☐ Get 50 µl cDNA samples from ice. Add 20 µl ER-AT MM. Mix **>** spin.
- ☐ Place in thermal cycler and press play/continue.
- ☐ At 4 °C Hold step, remove from cycler **>** keep on ice.

Step 3: Ligate MBC-tagged adaptors

40 min

- ☐ Set up thermal cycler program (Table 7). Start and pause program.

Table 7: Thermal cycler program for Ligation (vol 100 µl)

Step	Temperature	Time
Step 1	20 °C	30 min
Step 2	4 °C	Hold

- ☐ Get 70 µl DNA samples from ice. Add 25 µl Lig MM from Step 1. Mix **>** spin.
- ☐ ● Add 5 µl XT HS2 RNA Adaptor Oligo Mix. Mix **>** spin.
- ☐ Place in thermal cycler and press play/continue.
- ☐ At 4 °C Hold step, remove from cycler **>** keep on ice.

Prep Ahead: Put AMPure XP beads at RT for use in next step (30 min equilibration)

Step 4: Clean-up ligation

30 min

- ☐ Get AMPure XP beads held at RT, vortex until homogeneous.
- ☐ Add 80 µl of AMPure XP beads to each cDNA sample well. Mix **>** incubate at RT 5 min.
- ☐ Collect beads using magnet (5–10 min) **>** remove and discard supernatant.
- ☐ Wash beads 2X with 200 µl freshly-prepared 70% ethanol per wash.
  Washes completed: ☐ 1 ☐ 2
- ☐ After second wash solution removed, spin briefly **>** remove residual ethanol.
- ☐ Dry samples unsealed on the thermal cycler at 37 °C (1–2 min).
- ☐ Elute by adding 35 µl nuclease-free water to each sample well. Seal wells **>** vortex **>** brief spin. Incubate at RT 2 min.
- ☐ Collect beads using magnet (~5 min).
- ☐ Transfer 34 µl cleared supernatant to fresh well **>** keep on ice.

Note: Reagent vial cap colors are indicated by colored circles adjacent to reagent use instructions. Clear cap is indicated by "c".

Step 5: Amplify and dual index (Pre-capture PCR)

35 min

☐ Set up thermal cycler program (Table 8). Start and pause program.

Table 8: Thermal cycler program for pre-capture PCR (vol 50 µl)

Step	Number of Cycles	Temperature	Time
Step 1	1	98 °C	2 min
Step 2	**For high-quality RNA input libraries:**		
	☐ 10 for 100–200 ng RNA input	98 °C	30 sec
	☐ 11 for 50 ng RNA input	60 °C	30 sec
	☐ 12 for 10 ng RNA input		
	For good-quality FFPE RNA input libraries:		
	☐ 12 for 100–200 ng RNA input	72 °C	1 min
	☐ 13 for 50 ng RNA input		
	☐ 14 for 10 ng RNA input		
	For poor-quality FFPE RNA input libraries:		
	☐ 13 for 100-200 ng RNA input		
	☐ 14 for 50 ng RNA input		
Step 3	1	72 °C	5 min
Step 4	1	4 °C	Hold

☐ Prepare Pre-capture PCR master mix (Pre-PCR MM; Table 9).
Mix **>** spin **>** keep on ice.

Table 9: Pre-PCR MM reagent volumes (including excess for supported run sizes)

Reagent		16 Rxn Kits		96 Rxn Kits	
	Per Rxn	8 Rxn	16 Rxn	24 Rxn	96 Rxn
ⓒ 5X Herculase II Buffer with dNTPs	10 µl	90 µl	180 µl	260 µl	1000 µl
● Herculase II Fusion DNA Polymerase	1 µl	9 µl	18 µl	26 µl	100 µl
Total	11 µl	99 µl	198 µl	286 µl	1100 µl

☐ Get 34 µl purified library samples from ice. Add 11 µl Pre-PCR MM.

☐ Add 5 µl of the appropriate SureSelect XT HS2 Index Primer Pair to each sample well. Vortex **>** spin.

☐ Resume thermal cycler program without adding samples. Once cycler reaches 98 °C, add sample plate/strip **>** close lid.

Prep Ahead: Put AMPure XP beads at RT for use in next step (30 min equilibration)

Step 6: Clean-up library

30 min

☐ Get AMPure XP beads held at RT, vortex until homogeneous.

☐ Remove samples from cycler **>** add 50 µl of AMPure XP beads to each amplified DNA well. Mix **>** incubate at RT 5 min.

☐ Collect beads using magnet (5 min) **>** remove and discard supernatant.

☐ Wash beads 2X with 200 µl freshly-prepared 70% ethanol per wash.
Washes completed: ☐ 1 ☐ 2

☐ After second wash solution removed, spin briefly **>** remove residual ethanol.

☐ Dry samples unsealed on the thermal cycler at 37 °C (1–2 min).

☐ Elute by adding 15 µl nuclease-free water to each sample well.
Seal wells **>** vortex **>** brief spin. Incubate at RT 2 min.

☐ Collect beads using magnet (2–3 min).

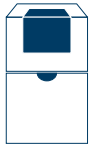
☐ Transfer cleared supernatant to fresh well **>** keep on ice.

Step 7: Quality control

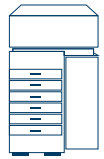
Varies

Assess quality and quantity using one of these platforms:

Agilent 2100 Bioanalyzer System with DNA 1000 Assay



Agilent 4200 or 4150 TapeStation System with D1000 Assay



Agilent 5200, 5300 or 5400 Fragment Analyzer System with NGS Fragment Kit (1–6000 bp)

Stopping point

Hybridization/Capture

Note: Reagent vial cap colors are indicated by colored circles adjacent to reagent use instructions.

Step 1: Hybridize libraries to probe

1.5–2.5 hrs/overnight

☐ Set up thermal cycler program (Table 10). Start and pause program.

Table 10: Thermal cycler program for hybridization (vol 30 µl; heated lid ON)

Step	Probe	Number of Cycles	Temperature	Time
Step 1	All probes	1	95 °C	5 min
Step 2	All probes	1	65 °C	10 min
Step 3	All probes	1	65 °C	1 min *PAUSE HERE* *Resume after adding probe*
Step 4	All probes	60	65 °C[1]	1 min
			37 °C	3 sec
Step 5	☐ XT HS Human All Exon V8 or ☐ All other probes	1	65 °C	60 min
			65 °C	Hold briefly[2]
		1	65 °C[1]	Hold briefly[2]

Hybridization program variation footnotes:

1. Hybridization at 65 °C (steps 4 and 5) is optimal for XT HS designs. Optimal temperature may be lower for some legacy XT probe designs.
2. **For overnight workflow**, replace the final brief hold at 65 °C with hold at 21 °C for up to 16 hours.

☐ Place prepared cDNA libraries in wells: 200 ng DNA brought to 12 µl with nuclease-free water for each sample.

● Add 5 µl SureSelect XT HS2 Blocker Mix to sample wells. Seal wells **>** vortex 5 sec **>** brief spin **>** verify absence of bubbles in wells.

☐ Place in thermal cycler and press play/continue. **Run through Step 2, pausing cycler at Step 3**. During cycler Steps 1–2, do reagent prep tasks below.

☐ Prepare 25% RNase Block solution (see Table 11). Mix **>** keep on ice.

Table 11: 25% RNase Block prep volumes (including excess for supported run sizes)

Reagent		16 Rxn Kits		96 Rxn Kits	
	Per Rxn	8 Rxn	16 Rxn	24 Rxn	96 Rxn
● SureSelect RNase Block	0.5 µl	4.5 µl	9 µl	12.5 µl	50 µl
Nuclease-free water	1.5 µl	13.5 µl	27 µl	37.5 µl	150 µl
Total	2 µl	18 µl	36 µl	50 µl	200 µl

☐ Prepare the Probe Hyb Mix needed for your probe design. Use Table 12 for designs ≥3 Mb or use Table 13 for designs <3 Mb. Vortex **>** spin **>** use immediately (keep briefly at RT while adding to hyb).

Step 1 Continued

Table 12: Probe Hyb Mix for probes ≥3 Mb (includes excess for supported run sizes)

Reagent		16 Rxn Kits		96 Rxn Kits	
	Per Rxn	8 Rxn	16 Rxn	24 Rxn	96 Rxn
25% RNase Block (table 11)	2 µl	18 µl	36 µl	50 µl	200 µl
Probe **(≥3 Mb design)**	5 µl	45 µl	90 µl	125 µl	500 µl
SureSelect Fast Hybridization Buffer	6 µl	54 µl	108 µl	150 µl	600 µl
Total	13 µl	117 µl	234 µl	325 µl	1300 µl

Table 13: Probe Hyb Mix for probes <3 Mb (includes excess for supported run sizes)

Reagent		16 Rxn Kits		96 Rxn Kits	
	Per Rxn	8 Rxn	16 Rxn	24 Rxn	96 Rxn
25% RNase Block (table 11)	2 µl	18 µl	36 µl	50 µl	200 µl
Probe **(<3 Mb design)**	2 µl	18 µl	36 µl	50 µl	200 µl
SureSelect Fast Hybridization Buffer	6 µl	54 µl	108 µl	150 µl	600 µl
Nuclease-free water	3 µl	27 µl	54 µl	75 µl	300 µl
Total	13 µl	117 µl	234 µl	325 µl	1300 µl

☐ With thermal cycler paused at Step 3, add 13 µl Probe Hyb Mix to each sample well. Pipette 8–10X to mix **>** cap wells with domed caps.
Wells must be fully sealed to prevent evaporation.

☐ Brief vortex **>** brief spin **>** verify absence of bubbles in wells. Immediately return samples to cycler **>** press play/continue to run hybridization.

Step 2: Prepare streptavidin beads

30 min

For same-day hyb and capture, begin steps below ~30 min prior to completion of hybridization program in Table 10. For overnight workflow option, begin at start of Day 2.

☐ Get SureSelect Streptavidin Beads or Dynabeads MyOne Streptavidin T1 Beads from storage at 4 °C, vortex until homogeneous.

☐ Place 50 µl of streptavidin beads in each sample well of fresh plate/strip.

☐ Wash beads 3X with SureSelect Binding Buffer at RT. For each wash:

- ☐ Add 200 µl Binding Buffer per well of beads **>** mix well **>** spin briefly.
- ☐ Collect beads using magnet (5 min) **>** remove and discard supernatant.

Washes completed: ☐ 1 ☐ 2 ☐ 3

☐ Resuspend washed beads in 200 µl SureSelect Binding Buffer **>** keep at RT.

Note: Reagent vial cap colors are indicated by colored circles adjacent to reagent use instructions. Clear cap is indicated by "c".

Step 3: Capture hybridized libraries

70 min

- ☐ Once streptavidin beads prepared and hybridization program reaches final hold, transfer samples briefly to RT.
- ☐ Immediately transfer hyb reactions (~30 µl) to wells containing 200 µl washed beads. Pipette 5–8X to mix **>** seal wells with fresh domed caps.
- ☐ Incubate on a 96-well plate mixer at 1400–1900 rpm for 30 min at RT.
- ☐ During 30 min hyb capture, prep pre-warmed SureSelect Wash Buffer **2** (6 aliquots per library) for post-capture washes:
  - ☐ Place 200 µl Wash Buffer **2** in fresh plate/strip wells.
  - ☐ Seal wells **>** place in thermal cycler held at 70 °C.
- ☐ After 30 min hyb capture, spin samples briefly **>** collect beads using magnet (until solution clear) **>** remove and discard supernatant.
- ☐ Resuspend capture beads at RT in 200 µl SureSelect Wash Buffer **1** by pipetting up and down 15–20X or until beads fully resuspended.
- ☐ Collect capture beads using magnet (~1 min) **>** remove and discard supernatant.
- ☐ Remove from magnet **>** keep at RT. Wash capture beads 6X using the pre-warmed SureSelect Wash Buffer **2** aliquots held at 70 °C. For each wash:
  - ☐ Add 200 µl Wash Buffer **2** at 70 °C to beads in wells.
  - ☐ Pipette up and down 15–20X.
  - ☐ Seal wells **>** vortex 8 sec **>** spin briefly (do not pellet).
  - ☐ Incubate 5 min at 70 °C in thermal cycler.
  - ☐ Collect beads using magnet (1 min) at RT **>** remove and discard supernatant.

  Washes completed: ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6
- ☐ After all wash buffer removed from final wash, add 25 µl nuclease-free water per well **>** pipette up and down 8X **>** keep on ice.

Captured DNA remains on the streptavidin beads for post-capture amplification.

Step 4: Amplify enriched libraries (Post-capture PCR)

30 min

- ☐ Set up thermal cycler program (Table 14). Start and pause program.

Table 14: Thermal cycler program for post-capture PCR (vol 50 µl)

Step	Number of Cycles	Temperature	Time
Step 1	1	98 °C	2 min
Step 2	☐ 12 for probe designs >5 Mb ☐ 13 for probe designs 3–5 Mb ☐ 14 for probe designs 0.2–3 Mb ☐ 16 for probe designs <0.2 Mb	98 °C 60 °C 72 °C	30 sec 30 sec 1 min
Step 3	1	72 °C	5 min
Step 4	1	4 °C	Hold

- ☐ Prepare Post-capture PCR master mix (Post-PCR MM; Table 15). Mix **>** spin **>** keep on ice.

Table 15: Post-PCR MM reagent volumes (including excess for supported run sizes)

Reagent		16 Rxn Kits		96 Rxn Kits	
	Per Rxn	8 Rxn	16 Rxn	24 Rxn	96 Rxn
Nuclease-free water	13 µl	117 µl	234 µl	338 µl	1300 µl
ⓒ 5X Herculase II Buffer with dNTPs	10 µl	90 µl	180 µl	260 µl	1000 µl
● Herculase II Fusion DNA Polymerase	1 µl	9 µl	18 µl	26 µl	100 µl
ⓒ SureSelect Post-Capture Primer Mix	1 µl	9 µl	18 µl	26 µl	100 µl
Total	25 µl	225 µl	450 µl	650 µl	2500 µl

- ☐ Get 25 µl captured library bead suspensions from ice. Add 25 µl Post-PCR MM.
- ☐ Mix by pipetting until beads are in homogeneous suspension (Do **not** spin).
- ☐ Place samples in cycler **>** close lid **>** press play/continue.
- ☐ At 4 °C Hold step, remove from cycler **>** brief spin.
- ☐ Collect streptavidin beads using magnet (2 min).
- ☐ **Transfer supernatant (~50 µl) to well of fresh plate or strip >** keep on ice.

Prep Ahead: Put AMPure XP beads at RT for use in next step (30 min equilibration)

Step 5: Clean-up final libraries

30 min

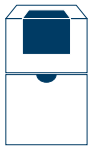
- ☐ Get AMPure XP beads held at RT, vortex until homogeneous.
- ☐ Add 50 µl of AMPure XP beads to each amplified DNA well **>** mix until beads in homogeneous suspension. Incubate at RT 5 min.
- ☐ Collect beads using magnet (3–5 min) **>** remove and discard supernatant.
- ☐ Wash beads 2X with 200 µl freshly-prepared 70% ethanol per wash.
  Washes completed: ☐ 1 ☐ 2
- ☐ After second wash solution removed, spin briefly **>** remove residual ethanol.
- ☐ Dry samples unsealed on the thermal cycler at 37 °C (1–2 min).
- ☐ Elute by adding 25 µl 1X Low TE to each sample well.
  Seal wells **>** vortex **>** brief spin. Incubate at RT 2 min.
- ☐ Collect beads using magnet (~2 min).
- ☐ Transfer cleared supernatant to fresh well **>** keep on ice.

Step 6: Quality control

Varies

Assess quality and quantity using one of these platforms:

Agilent 2100 Bioanalyzer System with High Sensitivity DNA Assay



Agilent 4200 or 4150 TapeStation System with High Sensitivity D1000 Assay

Agilent 5200, 5300 or 5400 Fragment Analyzer System with HS NGS Fragment Kit (1–6000 bp)

Stopping point

Step 7: Pool libraries for multiplex NGS

30 min

- ☐ Calculate the number of indexes that can be combined per lane, according to sequencer capacity and amount of sequencing data required per sample.
- ☐ Combine the libraries such that each index-tagged sample is present in equimolar amounts in the pool using one of the following methods:
  - ☐ Dilute each sample to be pooled to the concentration of the most dilute sample using Low TE **>** combine equal volumes of all samples in final pool.
  - ☐ Combine the appropriate volume of each sample to achieve equimolar concentration in the pool, then adjust the pool to the desired final volume using Low TE. See the **assay user guide** for more information on this method.

Run notes

Run Date	
Operator	
Probe Name or Design ID	
Index Pairs	
Reagent Lot Information	– cDNA Module – Library Prep Kit: – Target Enrichment Kit: – Probe: – Other Materials:
Sample Information	
Library Pooling Information	
Comments	

G9989-90500 Rev A0, July 2022

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