Preparing Kinnex™ libraries using Kinnex single-cell RNA kit

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing single-cell Kinnex libraries from 10x Chromium 3' or 5' cDNA using the *Kinnex single-cell RNA kit* (103-072-200) for library prep and sequencing on PacBio® Sequel® II, Sequel IIe, and Revio™ systems.

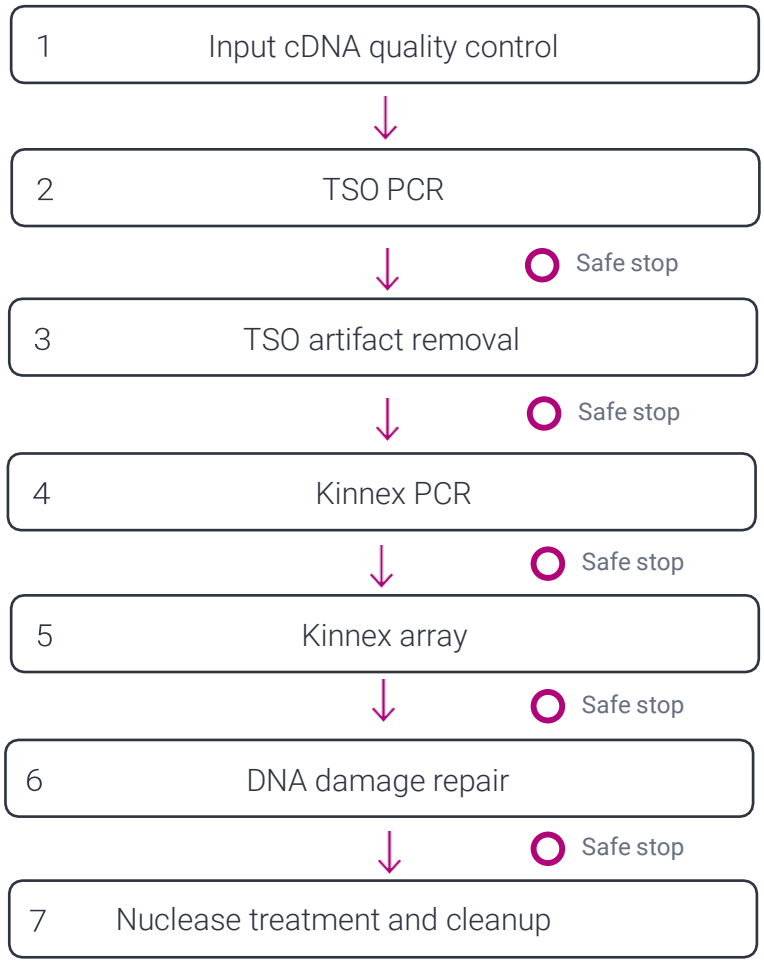
This kit is intended for use with single-cell cDNA generated using the *10x Chromium Next GEM Single Cell 3' kit v3.1* or *10x Chromium Next GEM Single Cell 5' kit v2*, standard throughput. It has not been tested for use on low throughput (LT) or high throughput (HT) kits which are currently unsupported.

Overview	
Samples per kit	12
Workflow time	3 days for up to 12 samples

cDNA input	
Quantity	>15 ng per library cDNA concentration should be >1ng/µL with up to 15 µL in volume. See step 2.1 for 10x cDNA input requirement.
Average segment lengths	500–1,000 bp
Average 16-segment array lengths	10–15 kb

103-254-300 REV 02 MAR2024

Workflow



Required materials and equipment

DNA sizing	
Femto Pulse system	Agilent Technologies, Inc. M5330AA
Femto Pulse gDNA 165 kb analysis kit	Agilent Technologies, Inc. FP-1002-0275
Agilent 2100 Bioanalyzer system	Agilent Technologies, Inc. G2939BA
Agilent High Sensitivity DNA Kit	Agilent Technologies, Inc. 5067-4627
DNA quantitation	
Qubit fluorometer	ThermoFisher Scientific Q33238
Qubit 1X dsDNA HS assay kit	ThermoFisher Scientific Q33230
SMRTbell® library preparation	
Kinnex™ single-cell concatenation kit	PacBio® 103-242-000*
Kinnex™ capture beads kit (12 rxn) (Includes Kinnex™ capture beads, Kinnex™ bead binding buffer, Kinnex™ bead washing buffer)	PacBio® 103-076-000*
SMRTbell® cleanup beads	PacBio® 102-158-300*
Elution buffer (50 mL)	PacBio® 101-633-500*
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS
8-channel pipettes	Any MLS
Wide orifice tips (200µL)	Rainin 30389241
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Microcentrifuge	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	V&P Scientific VP 772F4-1
Magnetic bead rack	ThermoFisher Scientific 12321D
Thermocycler compatible with 0.2 mL 8-tube strips	Any MLS
Tube rotator	Any MLS
1.5 mL DNA LoBind tubes	Eppendorf 022431021
0.5 mL DNA LoBind tubes	Eppendorf 022431005

*Sold as part of bundle *Kinnex™ single-cell RNA kit* (103-072-200)

General best practices

Safety precautions

Refer to the Safety Data Sheet (SDS) for information on reagent hazards and protocols for safe handling, use, storage, and disposal.

cDNA Input

- Optimal range of 3,000–10,000 target cell recovery from the 10x Chromium 3′ or 5′ single cell workflow.
- Follow the best practices in the 10x Chromium user guide up until cDNA amplification, cleanup and QC (refer to 10x Chromium user guide for 5′ kits and 3′ kits). Input cDNA quality control is highly recommended before proceeding to the Kinnex workflow.

Reagent and sample handling

- Room temperature is defined as any temperature in the range of 18–23°C for this protocol.
- Thaw the repair buffer, nuclease buffer, Kinnex ligase buffer, Kinnex adapter mix, and primers at room temperature.
- Briefly vortex reagent buffers and Kinnex adapters prior to use. Enzyme mixes do not require vortexing.
- Quick spin all reagents in a microcentrifuge to collect liquid at tube bottom prior to use.
- Keep all temperature-sensitive reagents on ice.

Temperature-sensitive reagents

Step used	Tube color	Reagent
TSO PCR and Kinnex PCR	Green	Kinnex single-cell PCR mix 103-244-500
	Yellow	Kinnex 3′ capture primer mix 103-182-400
	Red	Kinnex 5′ capture primer mix 103-182-200
	Orange	Kinnex primers premix (A-PQ)
		103-107-800 A
		103-107-900 B
		103-108-000 C
		103-108-100 D
		103-108-200 E
		103-108-300 F
		103-108-400 G
		103-153-000 H
		103-153-100 I
		103-153-200 J
		103-153-300 K
		103-153-400 L
		103-153-500 M

		103-153-600 N 103-153-700 O 103-153-800 PQ
Kinnex array formation	Light green	Kinnex single-cell enzyme 103-243-800
	Yellow	Kinnex single-cell ligase 103-244-000
	White	Kinnex single-cell ligase buffer 103-244-100
	Red	Kinnex single-cell ligation additive 103-244-400
	Blue	Kinnex adapter mix bc01 103-109-600 bc02 103-109-700 bc03 103-109-800 bc04 103-109-900
DNA damage repair Nuclease treatment	Green	DNA repair mix 103-110-000
	Purple	Repair buffer 102-244-300
	Light green	Nuclease mix 103-110-100
	Light purple	Nuclease buffer 103-110-200

- Bring SMRTbell cleanup beads and Qubit 1X dsDNA HS reagents to room temperature for 30 minutes prior to use.
- Pipette-mix all bead binding and elution steps until beads are distributed evenly in solution.
- Wide-bore pipette tips help to minimize foaming specifically when resuspending Kinnex capture beads.
- Pipette-mix all library prep reactions by pipetting up and down 10 times or until fully resuspended.
- Samples can be stored at 4°C at all safe stopping points listed in the protocol.
- 1.5X SMRTbell cleanup is recommended before Kinnex array formation. If the cDNA contains smaller fragments

<200 bp, it is recommended to increase the SMRTbell cleanup ratio to 2X.

The Kinnex capture beads kit is not temperature-sensitive and contains the following contents:

Tube color	Reagent
Clear	Kinnex capture beads 102-144-900 Kinnex bead binding buffer 103-145-40 Kinnex bead washing buffer 103-145-500

Thermocycler programs

Program thermocycler(s) prior to beginning the protocol for the first time.

1. TSO PCR program (15 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	5
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

or TSO PCR program (60-75 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	3
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

2. TSO artifact removal program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

3. Kinnex PCR program

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	68°C	9
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

4. Kinnex primer digestion program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

5. Kinnex array ligation program

Heated lid set at 52°C

Step	Time	Temperature
1	60 min	42°C
2	Hold	4°C

6. DNA damage repair program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

7. Nuclease treatment program

Heated lid set at 47°C

Step	Time	Temperature
1	60 min	37°C
2	Hold	4°C

Workflow steps

1. Input cDNA quality control

This protocol requires at least 15 ng of 10x Chromium 3′ single cell cDNA. Follow the 10x Chromium user guide up until cDNA amplification, cleanup and QC (refer to 10x Chromium user guide for 5′ kits and 3′ kits). Before you begin, evaluate the quantity and size distribution of input cDNA to determine whether it is suitable for the protocol (average size between 500–1500 bp).

✔	Step	Instructions
	1.1	Bring the Qubit 1X dsDNA HS working solution and standards to room temperature.
	1.2	Pulse vortex or pipette mix each sample to homogenize the DNA in solution.
	1.3	Quick spin each sample to collect liquid.
	1.4	Take a 1 µL aliquot from each sample.
	1.5	Measure DNA concentration with a Qubit fluorometer using the 1X dsDNA HS kit.
	1.6	Dilute each sample to 1.0-1.5 ng/µL in elution buffer or water, based on the Qubit reading.
	1.7	Measure DNA size distribution with a Bioanalyzer system using the High Sensitivity DNA Kit.
	1.8	Proceed to the next step of the protocol if sample quality is acceptable.

2. TSO PCR

This PCR step generates biotinylated DNA-fragments to enable removal of TSO priming artifacts generated during cDNA synthesis.

cDNA amplification with Kinnex capture primers:

✔	Step	Instructions
	2.1	Normalize cDNA sample input to 15 ng if it is between 15 ng and 59 ng using elution buffer. Normalize cDNA sample input to 75 ng if it is higher than 75 ng using elution buffer. For cDNA amounts between 60–75ng, proceed without normalizing. Select either the Kinnex 3' or 5' capture primer mix depending on the 10x Genomics kit used. Set up the following PCR reaction on ice (RM1). **Reaction Mix 1 (RM1):**

✔	Tube color	Component	Volume
		Nuclease-free water	Up to 50 µL
	Green	Kinnex single-cell PCR mix (103-244-500)	25 µL
	Red	Kinnex 5′ capture primer mix (103-182-200)	10 µL
		or	

	Yellow	Kinnex 3' capture primer mix (103-182-400)	
		10x 5' or 3' cDNA library (1–5 ng/µL)	Up to 15 µL
		Total volume	**50 µL**

2.2 Pipette-mix **RM1**.

2.3 Quick spin **RM1** in a microcentrifuge to collect liquid.

2.4 Select the TSO PCR program based on cDNA input. Keep sample on ice until thermal cycler lid has heated to 105°C.

TSO PCR program (15 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	5
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

or TSO PCR program (60–75 ng input)

Heated lid set at 105°C

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	65°C	3
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Cleanup with 1.5X SMRTbell cleanup beads

2.5 Add 1.5X v/v (75 µL) of resuspended, room-temperature SMRTbell cleanup beads to each tube of amplified cDNA.

2.6 Pipette mix the beads until evenly distributed.

2.7 Quick spin the tube strip in a microcentrifuge to collect liquid.

2.8 Leave at room temperature for 10 minutes to allow DNA to bind beads.

2.9 Place tube strip in a magnetic separation rack until beads separate fully from the solution.

2.10 Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.

2.11	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
2.12	Repeat the previous step.
2.13	Remove residual 80% ethanol: • Remove tube strip from the magnetic separation rack. • Quick spin tube strip in a microcentrifuge. • Place tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
2.14	Remove tube strip from the magnetic rack. Immediately add 42 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
2.15	Quick spin the tube strip in a microcentrifuge to collect liquid.
2.16	Leave at room temperature for 5 minutes to elute DNA.
2.17	Place tube strip in a magnetic separation rack until beads separate fully from the solution.
2.18	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard old tube strip with beads.
2.19	Recommended: Evaluate sample concentration. Take a 1 µL aliquot from each tube. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Yield typically ranges from 100–600 ng.
2.20	Proceed to the next step of the protocol if sample quantity is acceptable (between 100 ng – 600 ng).
	SAFE STOPPING POINT – Store at 4°C

3. TSO artifact removal

In this step, removal of DNA fragments containing TSO artifacts is performed using Kinnex capture beads. A tutorial video demonstrating this step is available.

✔	Step	Instructions
	3.1	Bring Kinnex capture beads kit to room temperature. Resuspend the beads by vortexing.
	3.2	Transfer 10 µL resuspended Kinnex capture beads per sample to a PCR tube. Scale up the amount of beads if processing more than 4 samples (with 10% overage). If preparing more than 40 µL of beads, use a 1.5 mL LoBind tube instead of PCR tube.
	3.3	Place the tube on the magnet until the beads separate fully from the solution.
	3.4	Carefully remove and discard the supernatant while the tube remains on the magnet. Avoid touching the bead pellet with the pipette tip.
	3.5	• Remove the tube from the magnet. • Add 40 µL Kinnex bead binding buffer along the inside wall of the tube where the beads are collected and gently resuspend by pipetting using wide bore tips. DO NOT VORTEX. Note: the solution may be viscous. Highly recommend using wide bore tips to avoid foaming. When excess bubbles are present, lower cDNA recovery is expected. • Quick-spin the tube in a microcentrifuge if needed. Note: Scale up the volume of Kinnex capture binding buffer accordingly if preparing more than 40 µL of beads.
	3.6	Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.
	3.7	• Resuspend the beads in 40 µL Kinnex bead binding buffer by pipetting slowly using wide bore tips. DO NOT VORTEX. Note: the solution may be viscous. Highly recommend using wide bore tips to avoid foaming. When excess bubbles are present, lower cDNA recovery is expected. Note: Scale up the volume of Kinnex capture binding buffer accordingly, if preparing more than 40 µL of beads. • Distribute 40 µL of resuspended Kinnex capture beads into the appropriate number of PCR tubes before proceeding to Step 3.8.
	3.8	Add 40 µL of a solution containing the biotinylated DNA-fragments (from Step 2.18) to the resuspended beads. Mix carefully using wide bore tips to avoid foaming of the solution.
	3.9	Incubate the tube at room temperature for 15 minutes on a rotator to keep the beads in suspension. Quick-spin the tube in a microcentrifuge to collect liquid.
	3.10	Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.
	3.11	Resuspend the Kinnex capture beads/DNA-complex in 80 µL Kinnex bead washing buffer by rigorous pipette mixing until evenly distributed.
	3.12	Place the tube on the magnet until the beads separate fully from the solution and remove the

supernatant.

3.13	Remove the tube from the magnet. Resuspend the Kinnex capture beads/DNA-complex in 80 µL Kinnex bead washing buffer by rigorous pipette mixing until evenly distributed.
3.14	Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.
3.15	Remove the tube from the magnet. Resuspend the Kinnex capture beads/DNA complex in 80 µL nuclease free water by pipette mixing until evenly distributed.
3.16	Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.
3.17	Resuspend the capture beads/DNA-complex in 40 µL of elution buffer by pipette mixing until evenly distributed.
3.18	Add 2 µL Kinnex enzyme to the sample with capture beads to cleave the captured DNA products from Kinnex capture beads.
3.19	Pipette-mix each sample and a very quick spin in a microcentrifuge to collect liquid.
3.20	Run the TSO artifact removal program. **TSO artifact removal program** Heated lid set at 47°C Step 1: 30 min at 37°C Step 2: Hold at 4°C
3.21	Place the tube on the magnet for 1 minute and move the supernatant containing the library to a fresh tube.
3.22	Cleanup with 1.5X SMRTbell cleanup beads.
3.23	Add 1.5X v/v (63 µL) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
3.24	Pipette-mix the beads until evenly distributed.
3.25	Quick-spin the tube strip in a microcentrifuge to collect liquid.
3.26	Leave at room temperature for 10 minutes to allow DNA to bind beads.
3.27	Place tube strip in a magnetic separation rack until the beads separate fully from the solution.
3.28	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
3.29	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
3.30	Repeat the previous step.
3.31	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge.

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

	• Place the tube strip back in a magnetic separation rack until the beads separate fully from the solution. Pipette off residual 80% ethanol and discard.
3.32	Remove the tube strip from the magnetic rack. Immediately add 46 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
3.33	Quick-spin the tube strip in a microcentrifuge to collect liquid.
3.34	Leave at room temperature for 5 minutes to elute DNA.
3.35	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
3.36	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube. Discard the old tube strip with beads.
3.37	Recommended: Evaluate sample concentration. • Take a 1 µL aliquot from each tube. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
3.38	Proceed to the next step of the protocol if sample quantity is acceptable (minimum 25 ng).
	SAFE STOPPING POINT – Store at 4°C

4. Kinnex PCR

Perform 16 parallel cDNA amplification reactions with Kinnex primers to generate DNA fragments containing orientation-specific Kinnex segmentation adapter sequences.

Note: All 16 PCR reactions are required. Missing/incorrect primer pairs will result in no/low SMRTbell yield.

✔	Step	Instructions
	4.1	Set up the following PCR reaction mix per sample on ice. Reaction Mix 2 (RM2):

✔	Master mix components	Volume for 16X concatenation*
	PCR-grade water	176–X µL
	Kinnex single-cell PCR mix (103-244-500)	220 µL
	25 ng of amplified cDNA from Step 3.38	X µL
	Total volume	396 µL

X = 25 (ng) / purified pooled cDNA concentrations from Step 3.38
*10% overage included

4.2	Quick-spin RM2 in a microcentrifuge to collect liquid.
4.3	Add 22.5 µL of RM2 to a new PCR tube on ice. Repeat this step to prepare a total of 16 tubes per

sample (each containing 22.5 µL of RM2).

4.4 Add 2.5 µL of Kinnex primers premix into each of 16 PCR tubes on ice according to the table below.

PCR tube	Kinnex primers premix	PN
1	Kinnex primers premix A	103-107-800
2	Kinnex primers premix B	103-107-900
3	Kinnex primers premix C	103-108-000
4	Kinnex primers premix D	103-108-100
5	Kinnex primers premix E	103-108-200
6	Kinnex primers premix F	103-108-300
7	Kinnex primers premix G	103-108-400
8	Kinnex primers premix H	103-153-000
9	Kinnex primers premix I	103-153-100
10	Kinnex primers premix J	103-153-200
11	Kinnex primers premix K	103-153-300
12	Kinnex primers premix L	103-153-400
13	Kinnex primers premix M	103-153-500
14	Kinnex primers premix N	103-153-600
15	Kinnex primers premix O	103-153-700
16	Kinnex primers premix PQ	103-153-800

4.5 Pipette-mix each sample. The total volume of each tube should be 25.0 µL.

4.6 Quick-spin the strip tubes in a microcentrifuge to collect liquid.

4.7 Run the Kinnex PCR program with heated lid set to 105°C. Keep sample on ice until thermal cycler lid has heated to 105°C.

Step	Time	Temperature	Cycles
1	3 min	98°C	1
2	20 sec	98°C	
3	30 sec	68°C	9
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Clean up with 1.5X SMRTbell cleanup beads.

4.8 Pool entire volume of all 16 reactions into a single 1.5 mL LoBind tube.

4.9 Add 1.5X v/v (600 µL) of resuspended, room-temperature SMRTbell cleanup beads to the PCR pool.

4.10 Pipette-mix the beads until evenly distributed.

4.11	Quick-spin the tube strip in a microcentrifuge to collect all liquid from the sides of the tubes.
4.12	Leave at room temperature for 10 minutes to allow DNA to bind beads.
4.13	Place 1.5mL LoBind tube in a magnetic separation rack until beads separate fully from the solution.
4.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
4.15	Slowly dispense 1 mL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
4.16	Repeat the previous step.
4.17	Remove residual 80% ethanol: • Remove the LoBind tube from the magnetic separation rack. • Quick-spin the LoBind tube in a microcentrifuge. • Place the LoBind tube back in a magnetic separation rack until beads separate fully from the solution. Pipette off residual 80% ethanol and discard.
4.18	Remove the LoBind tube from the magnetic rack. Immediately add 47 µL of elution buffer to each tube and resuspend the beads.
4.19	Quick-spin the LoBind tube in a microcentrifuge.
4.20	Incubate at room temperature for 5 minutes to elute DNA.
4.22	Place the LoBind tube in a magnetic separation rack until beads separate fully from the solution.
4.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new LoBind tube. Discard old tube with beads.
4.23	Recommended: Evaluate sample concentration. • Take a 1 µL aliquot from each tube, dilute with 9 µL of elution buffer. Using 1 µL of the dilution, measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit.
4.24	Proceed to the next step of the protocol if sample quantity is acceptable (maximum input: 5 µg). Do not proceed if less than 3 µg is available.

SAFE STOPPING POINT - Store at 4°C

5. Kinnex array formation

In this step, treat PCR amplified cDNA fragments with Kinnex enzyme to create single-stranded extensions to enable directional assembly of cDNA segments into a linear array.

✔	Step	Instructions
	5.1	In a 0.2 mL PCR tube, add **5 µg** of sample from Step 4.22, in 47 µL of volume (106 ng/µl). Dilute with elution buffer going into this step if sample is too concentrated.
	5.2	Add 10 µL of Kinnex enzyme to create single-stranded extensions on PCR-amplified cDNA fragments to enable subsequent directional assembly of 16 PCR products.
	5.3	Pipette-mix each sample.
	5.4	Run the Kinnex primer digestion program. **Kinnex primer digestion program** Heated lid set at 47°C Step 1: 30 min at 37°C Step 2: Hold at 4°C
	5.5	Add 3 µL of Kinnex adapter barcode 01–04 (use a single barcode per sample) and 20 µL of Kinnex ligation additive to each sample for a total volume of 80 µL. Blue: Kinnex barcode adapter — 3 µL Red: Kinnex ligation additive — 20 µL Total volume — 23 µL
	5.6	Pipette-mix each sample.
	5.7	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample in the order and volume listed below. Reaction Mix 3 (RM3): White: Kinnex single-cell ligase buffer 103-244-100 — 10 µL Yellow: Kinnex single-cell ligase 103-244-000 — 10 µL Total volume — 20 µL
	5.8	Pipette-mix **RM3**
	5.9	Quick-spin **RM3** in a microcentrifuge to collect liquid.
	5.10	Add 20 µL of **RM3** to each sample.
	5.11	Pipette-mix each sample .

Run the Kinnex array ligation program.

Kinnex array ligation program

5.12 Heated lid set at 52°C

Step	Time	Temperature
1	60 min	42°C
2	Hold	4°C

	Cleanup with 1.2X SMRTbell cleanup beads
5.13	Add 1.2X v/v (120 µL) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
5.14	Pipette-mix the beads until evenly distributed.
5.15	Quick-spin the tube strip in a microcentrifuge to collect liquid.
5.16	Leave at room temperature for 10 minutes to allow DNA to bind beads.
5.17	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
5.18	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
5.19	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
5.20	Repeat the previous step.
5.21	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. Pipette off residual 80% ethanol and discard.
5.22	Remove the tube strip from the magnetic rack. Immediately add 43 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
5.23	Quick-spin the tube strip in a microcentrifuge to collect liquid.
5.24	Leave at room temperature for 5 minutes to elute DNA.
5.25	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
5.26	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube. Discard old tube strip with beads.
5.27	Recommended: Evaluate sample concentration. • Take a 1 µL aliquot from each tube, dilute with 4 µL of elution buffer. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Proceed to DNA damage repair.
	SAFE STOPPING POINT – Store at 4°C

6. DNA damage repair

✔	Step	Instructions
	6.1	In a 0.2 mL PCR tube, add 42 µL of sample from Step 5.26
	6.2	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample in the order and volume listed below. Reaction Mix 4 (RM4):

✔	Tube color	Component	Volume
	Purple	Repair buffer 102-696-100	6 µL
	Green	DNA repair mix 102-696-000	2 µL
		Total volume	8 µL

	6.3	Pipette-mix **RM4**.
	6.4	Quick-spin **RM4** in a microcentrifuge to collect liquid.
	6.5	Add 8 µL of **RM4** to each sample. Total volume should equal 50 µL.
	6.6	Pipette-mix each sample.
	6.7	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	6.8	Run the DNA damage repair program.

DNA damage repair program

Heated lid set at 47°C

Step	Time	Temperature
1	30 min	37°C
2	Hold	4°C

		Clean up with 1.2X SMRTbell cleanup beads
	6.9	Add 1.2X v/v (60 µL) of resuspended, room-temperature SMRTbell cleanup beads to each sample.
	6.10	Pipette-mix the beads until evenly distributed.
	6.11	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	6.12	Leave at room temperature for 10 minutes to allow DNA to bind beads.
	6.13	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.14	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	6.15	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
	6.16	Repeat the previous step.

	6.17	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	6.18	Remove the tube strip from the magnetic rack. Immediately add 40 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	6.19	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	6.20	Leave at room temperature for 5 minutes to elute DNA.
	6.21	Place the tube strip in a magnetic separation rack until beads separate fully from the solution.
	6.22	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new tube strip. Discard old tube strip with beads.

SAFE STOPPING POINT - Store at 4°C

7. Nuclease treatment and cleanup

✔	Step	Instructions
	7.1	Add the following components in the order and volume listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. For individual preps, add components directly to each sample from the previous step in the order and volume listed below. Reaction Mix 5 (RM5): ✔ / Tube color / Component / Volume Light purple / Nuclease buffer 103-110-200 / 5 µL Light green / Nuclease mix 103-110-100 / 5 µL Total volume / 10 µL
	7.2	Pipette-mix **RM5**.
	7.3	Quick-spin **RM5** in a microcentrifuge to collect liquid.
	7.4	Add 10 µL of **RM5** to each sample. Total volume should equal 50 µL.
	7.5	Pipette-mix each sample.
	7.6	Quick-spin the strip tube in a microcentrifuge to collect liquid.

7.7 Run the nuclease treatment program.

Nuclease treatment program

Heated lid set at 47°C

Step	Time	Temperature
1	60 min	37°C
2	Hold	4°C

7.8 Add 60 µL SMRTbell cleanup beads to each sample from the previous step. Pipette-mix the beads until evenly distributed.

7.9 Quick-spin the tube strip in a microcentrifuge to collect all liquid.

7.10 Leave at room temperature for 10 minutes to allow DNA to bind beads.

7.11 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

7.12 Slowly pipette off the cleared supernatant without disturbing the beads. It is recommended to save the supernatant in another tube strip in case of poor DNA recovery.

7.13 Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.

7.14 Repeat the previous step.

7.15 Remove residual 80% ethanol:

- Remove tube strip from the magnetic separation rack.
- Quick spin tube strip in a microcentrifuge.
- Place tube strip back in a magnetic separation rack until beads separate fully from the solution.

Pipette off residual 80% ethanol and discard.

7.16 Remove the tube strip from the magnetic rack. Immediately add 26 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.

7.17 Quick-spin the tube strip in a microcentrifuge to collect liquid.

7.18 Leave at room temperature for 5 minutes to elute DNA.

7.19 Place the tube strip in a magnetic separation rack until beads separate fully from the solution.

7.20 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube. Discard old tube strip with beads.

7.21 Take a 1 µL aliquot from each tube. Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass.

Recommended: Further dilute each aliquot to 250 pg/µL with Femto Pulse dilution buffer. Measure final SMRTbell library size distribution with a Femto Pulse system.

7.22 Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing. **DNA concentration must be less than 20 ng/µl to go into ABC. Using a concentration above 20 ng/µl will result in lower loading during sequencing.**

7.23 Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release	01	October 2023
Updated to incorporate PCR program into workflow steps, clarify sample quantity in TSO artifact removal, and add required DNA concentration before sequencing	02	March 2024