Technical note

PREPARING KINNEX™ SINGLE-CELL LIBRARIES WITH TWIST EXOME ENRICHMENT KITS

Overview

This technical note describes the experimental conditions of a modified workflow preparing PacBio® **Kinnex single-cell libraries** with Twist exome enrichment kits. The purpose of using exome enrichment is to enrich for spliced mRNA in single-nuclei libraries[1]. The following workflow has been tested on *10x Single Cell 3' Gene Expression* (v3.1) libraries, but is also compatible with single-nuclei libraries generated with the following:

- *10x Single Cell Gene Expression 3'* (v3.1, v4)
- *10x Single Cell Immune Profiling 5'* (v2, v3)
- Parse Evercode WT and WT mini kits (see the **Parse technical note**)

The workflow has been tested with the following Twist Exome Enrichment Panels:

- Twist Human Comprehensive Exome Panel
- Twist RNA Exome Panel

NOTE: This is a single reaction workflow. This protocol does not support pooled indexed libraries such as those generated using **Twist's UDI Adapter Systems**.

Required materials

Please refer to the **Kinnex single-cell protocol** and respective single-cell provider manual for materials required.

Single-cell cDNA generation	
Single-nuclei cDNA library	Refer to single-cell vendor (10x, Parse)
10x cDNA primer*	10x 2000089
PacBio Kinnex library generation	
Kinnex single-cell RNA kit	Refer to **Kinnex single-cell protocol**
Custom 10x or Parse blockers†	Refer to Appendix 1
Twist exome enrichment protocol	
Twist Comprehensive Exome Panel **OR** **Twist RNA Exome Panel**	Twist 102031: 2 rxn Twist 102032: 12 rxn Twist 102033: 96 rxn Twist 107143: 2 rxn Twist 107144: 12 rxn Twist 107146: 96 rxn
Twist Universal Blockers	Twist 100856: 2 rxn Twist 100578: 12 rxn Twist 100767: 96 rxn
Twist Standard Hyb fand Wash Kit v2 with Amp Mix	Twist 105559: 2 rxn Twist 105560: 12 rxn Twist 105561: 96 rxn

* The 10x cDNA primer is included in *10x Single Cell Gene Expression* kits. If stock kit primers are not available, please refer to Appendix 1 for custom ordering.
† Custom blockers are required to replace the Twist Universal Blockers to enable Kinnex compatibility. Please refer to Appendix 2 for custom blockers.

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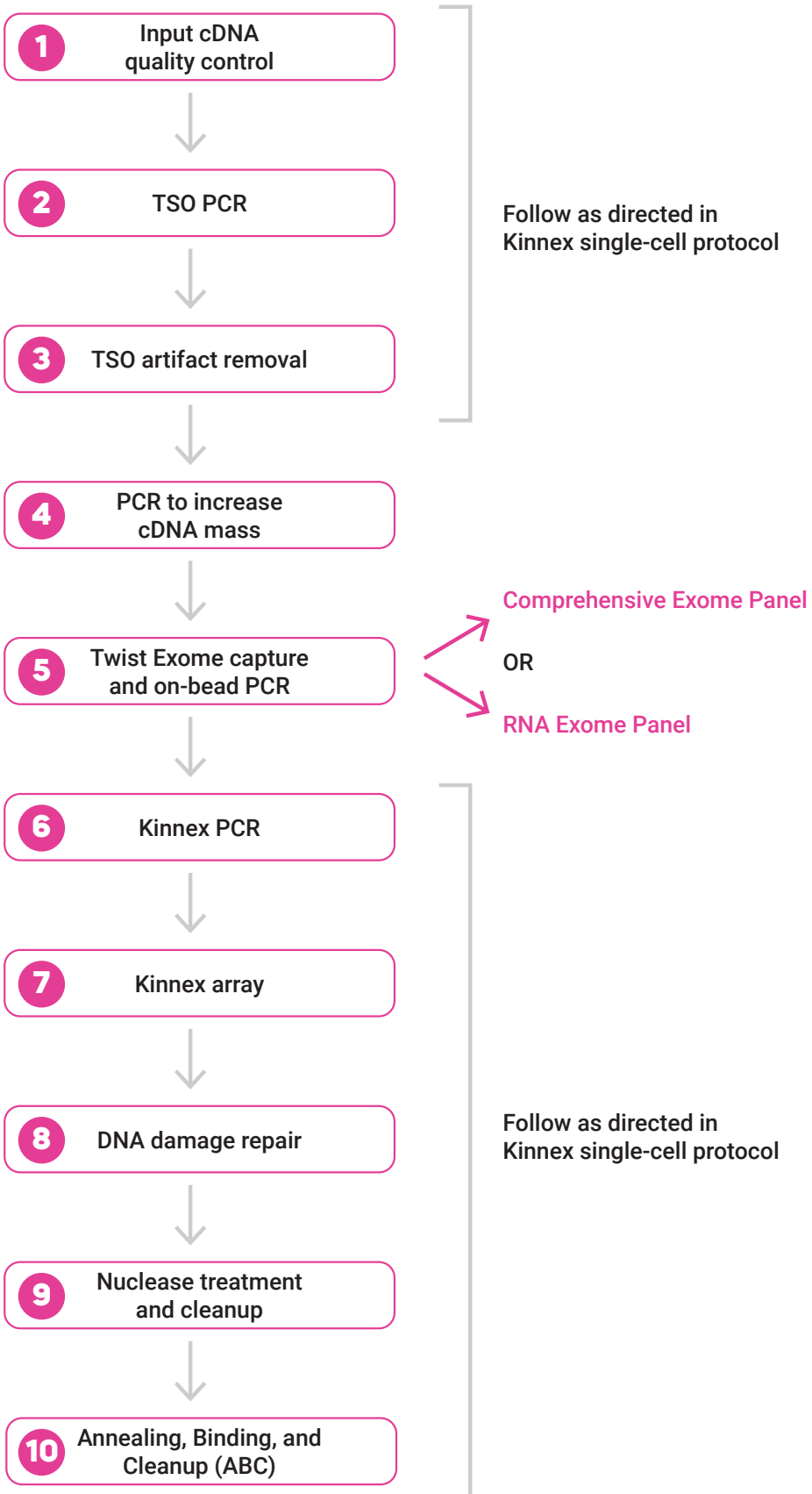
Workflow


1 Input cDNA quality control
2 TSO PCR
3 TSO artifact removal
Follow as directed in Kinnex single-cell protocol
4 PCR to increase cDNA mass
5 Twist Exome capture and on-bead PCR
Comprehensive Exome Panel
OR
RNA Exome Panel
6 Kinnex PCR
7 Kinnex array
8 DNA damage repair
9 Nuclease treatment and cleanup
10 Annealing, Binding, and Cleanup (ABC)
Follow as directed in Kinnex single-cell protocol

Library preparation

Follow Kinnex protocol instructions until the end of the TSO artifact removal step. If using compatible 10x products, follow the **Kinnex single-cell library protocol** through the end of step 3: TSO artifact removal. If using compatible Parse Biosciences products, follow the **Parse-Kinnex tech note** until the end of step 4.

Step 4: PCR to increase cDNA mass

This additional PCR step is required to generate sufficient cDNA mass for Twist exome enrichment (500 ng).

✓	Step	Instructions

Set up the following PCR reaction on ice (RM1).

Reaction Mix (RM1):

4.1.

✓	Component	Volume
	Eluted cDNA	Up to 30 µL
	Kinnex single-cell PCR mix	50 µL
	10x cDNA primer	20 µL
	Total volume	**100 µL**

4.2. Pipette-mix **RM1**.

4.3. Quick-spin **RM1** in a microcentrifuge to collect liquid.

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

NOTE: Tailor number of cycles to prevent over-cycling. Aim for 500–600 ng of yield.

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

Cleanup with 1.5X SMRTbell® cleanup beads

4.5. Add 1.5X v/v (150 µL) of resuspended, room-temperature SMRTbell cleanup beads to each tube of amplified cDNA.

4.6. Pipette-mix the beads until they are evenly distributed.

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

4.8. Incubate at room temperature for 10 minutes to allow DNA to bind to the beads.

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

4.10. Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.

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

4.12. Repeat the previous step.

4.13. 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 the beads separate fully from the solution.
- Remove residual 80% ethanol and discard.

4.14. 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.

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

4.16. Incubate at room temperature for 5 minutes to elute DNA.

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

4.18. Slowly aspirate the cleared eluate without disturbing the beads. Transfer the eluate to a new tube strip. Discard the old tube strip with beads.

4.19. 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.

4.20. Proceed to the next step of the protocol if the sample quantity is acceptable (500–600 ng). Repeat this section to generate sufficient yield if necessary.

SAFE STOPPING POINT—Store at 4°C

Step 5.1: Twist exome capture setup

The hybridization capture workflow below is a modification of the **Twist Targeted Enrichment Standard Hyb v2 Protocol.**

✓	Step	Instructions
		Dry down library from the end of step 4. The original Twist protocol recommends drying down 500 ng of cDNA using a speedvac (**step 1.4 of Twist protocol**). Alternatively, use SMRTbell cleanup beads as described below.
	5.1.1	Dry down cDNA with 1.5X SMRTbell cleanup beads.
	5.1.2	Elute 500 ng of cDNA from step 4.18 with Elution Buffer to achieve a total volume of 50 µL. Add 1.5X v/v (75 µL) of resuspended, room-temperature SMRTbell cleanup beads to each tube of amplified cDNA.
	5.1.3	Pipette-mix the beads until they are evenly distributed.
	5.1.4	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	5.1.5	Incubate at room temperature for 10 minutes to allow DNA to bind to the beads.
	5.1.6	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	5.1.7	Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.
	5.1.8	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, remove the 80% ethanol and discard.
	5.1.9	Repeat the previous step.
	5.1.10	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 the beads separate fully from the solution. • Remove residual 80% ethanol and discard.
	5.1.11	Remove the tube strip from the magnetic rack. Elute the cDNA from the beads by adding reagent mix 2 **(RM2)**. Pipette-mix the beads until they are evenly distributed.

Reaction Mix 2 (RM2):

✓	Component	Volume
	Twist Blocker solution	5 µL
	10x Custom Blocker Fwd **or** Parse Customer Blocker Fwd	2 µL
	10x or Parse Customer blocker Rev	2 µL
	Nuclease-free water	3 µL
	Total volume	**12 µL**

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

5.1.13 Incubate at room temperature for 5 minutes to elute DNA.

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

5.1.15 Slowly aspirate the cleared eluate without disturbing the beads. Transfer the eluate to a new tube strip. Discard the old tube strip with beads.

5.1.16 Proceed immediately to next section.

Step 5.2: Twist probe hybridization

This is the same step is described in step 2.2 of Twist protocol.

✓	Step	Instructions

5.2.1 Heat the Hybridization Mix at 65°C in the heat block for 10 minutes, or until all precipitate is dissolved. Cool to room temperature on the benchtop for 5 minutes

5.2.2 Prepare a probe solution in a PCR 0.2-mL strip tube with the following. Pipette-mix **RM3** until evenly distributed.

Reaction Mix 3 (RM3)

✓	Component	Volume
	Twist Hybridization Mix	20 µL
	Twist Comprehensive Exome Panel **or** Twist RNA Exome Panel	4 µL
	Nuclease-free water	Up to 4 µL
	Total volume	**28 µL**

5.2.3 Heat the probe solution from step 5.2.2 to 95°C for 2 min in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes.

5.2.4 While the probe solution is cooling on ice, heat the tube containing the resuspended cDNA library pool from step 5.1.15 at 95°C for 5 minutes in a thermal cycler with the lid at 105°C. Equilibrate both the probe solution and resuspended dsDNA library to room temperature for 5 minutes.

5.2.5 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended cDNA library pool. Mix well by vortexing.

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

5.2.7 Add 30 µL of Hybridization Enhancer to the top of the entire capture reaction.

5.2.8 Quick-spin the tube strip in a microcentrifuge to collect liquid. Ensure there are no bubbles present.

5.3.9 Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with the lid at 85°C.

Step 5.3: Bind hybridization capture reaction to Kinnex capture beads

This is a modified **step 3 of Twist protocol**.

✓	Step	Instructions
	5.3.0	Preheat the following tubes at 48°C until any precipitate is dissolved: • Twist Binding Buffer • Twist Standard Wash Buffer 1 • Twist Wash Buffer 2 For each hybridization reaction: • Equilibrate 800 µL Twist Binding Buffer to room temperature • Equilibrate 225 µL Twist Standard Wash Buffer 1 to 68°C • Leave 700 µL Twist Wash Buffer 2 at 48°C
	5.3.1	Bring the Kinnex capture beads kit to room temperature. Resuspend the Kinnex capture beads by vortexing.
	5.3.2	Transfer 100 µL resuspended Kinnex capture beads to a 1.5 mL LoBind tube.
	5.3.3	Place the tube on the magnet until the beads separate fully from the solution.
	5.3.4	Slowly aspirate and discard the supernatant while the tube remains on the magnet. Avoid touching the bead pellet with the pipette tip.
	5.3.5	• Remove the tube from the magnet. • Add 200 µL Twist 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. It is recommended to use 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.
	5.3.6	Place the tube on the magnet until the beads separate fully from the solution and remove the supernatant.
	5.3.7	Repeat step 5.3.5 and 5.3.6 two more times for a total of three washes. After removing the clear supernatant from the third wash, add a final 200 µL of Twist Binding Buffer and resuspend the beads by vortexing until homogenized.
	5.3.8	Heat the resuspended beads at 68°C for at least 10 min before next step.
	5.3.9	Rapidly transfer the contents of the overnight hybridization reaction from step 5.2.9 to the tubes containing the heated beads from step 5.3.8. NOTE: Do not let the reaction from the thermocycler cool to less than 70°C before transferring.
	5.3.10	Incubate for 5 minutes at 68°C. Agitation is not required. Do not vortex.
	5.3.11	Remove the tube containing the hybridization reaction with Kinnex capture beads from the mixer. Quick-spin the tube strip in a microcentrifuge to collect liquid.
	5.3.12	Place the tube on a magnetic stand for 1 minute.
	5.3.13	Remove and discard the clear supernatant including the Twist Hybridization Enhancer. Do not disturb the bead pellet. NOTE: It is acceptable to have some Twist Hybridization Enhancer visible after supernatant removal.
	5.3.14	Remove the tube from the magnetic stand and add 200 µL of 68°C Twist Standard Wash Buffer 1. Pipette-mix.
	5.3.15	Incubate the tube for 5 minutes at 68°C.
	5.3.16	Quick-spin the tube strip in a microcentrifuge to collect liquid.

5.3.17 Transfer the entire volume from step 5.3.16 into a new 1.5-mL LoBind tube. Place the tube on a magnetic stand for 1 minute.

5.3.18 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand and add 200 µL of 48°C Twist Wash Buffer 2. Pipette-mix. Quick-spin the tube strip in a microcentrifuge to collect liquid.

5.3.19 Incubate the tube for 5 min at 48°C.

5.3.20 Place the tube on a magnetic stand for 1 minute.

5.3.21 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

5.3.22 Repeat steps 5.3.18 to 5.3.21 two more times for a total of three washes.

After the final wash, use a 10 µL pipette to remove all traces of supernatant. Place the tube on ice and close lid.

Step 5.4: On-bead PCR amplification of Twist enriched library

This is a modified **step 4 of Twist protocol**.

✓	Step	Instructions

5.4.1 Set up the following PCR reaction on ice (RM4).

Reaction Mix 3 (RM4):

✓	Component	Volume
	Nuclease-free water	30 µL
	10x cDNA primers	20 µL
	Kinnex single-cell PCR mix	50 µL
	Total volume	**100 µL**

5.4.2 Pipette-mix **RM4.**

5.4.3 Quick-spin **RM4** in a microcentrifuge to collect liquid.

5.4.4 Program the thermal cycler to the following conditions. Keep sample on ice until the 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	65°C	5
4	4 min	72°C	
5	5 min	72°C	1
6	Hold	4°C	1

Cleanup with 1.5X SMRTbell cleanup beads

5.4.5 Add 1.5X v/v (150 µL) of resuspended, room-temperature SMRTbell cleanup beads to each tube of amplified cDNA.

5.4.6 Pipette-mix the beads until they are evenly distributed.

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

5.4.8 Incubate at room temperature for 10 minutes to allow DNA to bind to the beads.

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

5.4.10 Slowly remove the cleared supernatant without disturbing the beads. Discard the supernatant.

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

5.4.12 Repeat the previous step.

5.4.13 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 the beads separate fully from the solution.
- Remove residual 80% ethanol and discard.

5.4.14 Remove the 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.

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

5.4.16 Incubate at room temperature for 5 minutes to elute DNA.

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

5.4.18 Slowly aspirate the cleared eluate without disturbing the beads. Transfer eluate to a new tube strip. Discard old tube strip with beads.

5.4.19 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.

5.4.20 If the sample quantity is acceptable (25 ng is required), proceed to the Kinnex PCR step of the **Kinnex single-cell protocol** and complete the remainder of the library construction.

SAFE STOPPING POINT—Store at 4°C

Appendix 1

Order custom primers as described below from preferred oligo vendors. Standard desalt primers are acceptable. Use 1 µM of final concentration of each primer (10 µL FWD and 10 µL REV) in the PCR reaction.

10x cDNA primer FWD
CTACACGACGCTCTTCCGATCT
10x cDNA primer REV
AAGCAGTGGTATCAACGCAGAG

Appendix 2

Order custom primers as described below from preferred oligo vendors. An example of IDT code is below. The primers should be HPLC purified. Dilute the blockers to 10 µM and add 2 µL of each FWD and REV blocker.

Parse Custom Blocker FWD
CTACACGACGCTCTTCCGATCTCAGACGTGTGCTCTTCCGATC/3SpC3/
Parse Customer Blocker REV
AAGCAGTGGTATCAACGCAGAG/3SpC3/

10x Custom Blocker FWD
CTACACGACGCTCTTCCGATCT/3SpC3/
10x Custom Blocker REV
AAGCAGTGGTATCAACGCAGAG/3SpC3/

References

1. Hardwick, S. A., Hu, W., Joglekar, A., et al. (2022). Single-nuclei isoform RNA sequencing unlocks barcoded exon connectivity in frozen brain tissue. *Nature Biotechnology*, 40(7), 1082-1092. https://doi.org/10.1038/s41587-022-01231-3.

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