Library Preparation with the BD™ Precise WTA Single Cell Kit User's Guide

910000014 Rev. 02
11/2016

Becton, Dickinson and Company
BD Genomics
4040 Campbell Avenue
Menlo Park, CA 94025 USA
Tel 800.675.0908
techsupport.genomics@bd.com

Copyrights

© 2016, Becton, Dickinson and Company. All rights reserved. No part of this publication may be reproduced, transmitted, transcribed, stored in retrieval systems, or translated into any language or computer language, in any form or by any means: electronic, mechanical, magnetic, optical, chemical, manual, or otherwise, without prior written permission from BD Genomics.

The information in this guide is subject to change without notice. BD Genomics reserves the right to change its products and services at any time to incorporate the latest technological developments. Although this guide has been prepared with every precaution to ensure accuracy, BD Genomics assumes no liability for any errors or omissions, nor for any damages resulting from the application or use of this information. BD Genomics welcomes customer input on corrections and suggestions for improvement.

Trademarks

Agencourt and AMPure are registered trademarks of Beckman Coulter, Inc. Bioanalyzer is a registered trademark of Agilent Technologies. Eppendorf is a registered trademark of Eppendorf. MicroAmp is a registered trademark of Thermo Fisher Scientific.

BD, BD Logo, Molecular Indexing, and all other trademarks are property of Becton, Dickinson and Company. © 2016 BD

Patents

The BD™ Precise WTA Single Cell Kit is covered by one or more of the following US patents: 8835358, 9315857, 9290808, 9290809.

Regulatory information

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

History

Revision	Date	Change made
910000014 Rev. 02	11/2016	Initial release

Contents

Chapter 1: Introduction 5

About this guide 6

Safety symbols 6

Safety data sheets 7

Chapter 2: Getting Started 9

WTA Library preparation overview 10

Site requirements 14

Sample requirements and cell sorting recommendations 14

Thermal Cycler Programs Setup 15

Best practices 17

Chapter 3: Product information 19

Kits and storage conditions 20

Reagents 22

Consumables 22

Equipment 24

Chapter 4: Preparing a single cell library with the BD™ Precise WTA Single Cell Kit 27

Preparing the Precise WTA Single Cell Encoding Plate 28

Performing BD Precise reverse transcription 29

Pooling the samples 32

Purifying the pooled samples 32

Performing second strand (SS) synthesis on the pooled samples 35

Purifying the second strand synthesis products 37
Ligating the Precise WTA Adapter to the second strand synthesis products 39
Purifying the ligation products 41
Amplifying the ligation products 43
Purifying the WTA product 45
Performing quality control (QC) on the WTA product 47
Performing random primer extension 48
Purifying the primer extension product 51
Amplifying the primer extension product 53
Purifying the amplified library 55
Performing quality control on the amplified library 57
Sequencing the amplified library 58
References 59

Chapter 5: Troubleshooting 61

Sequencing 62

Chapter 6: Preparing a library with the BD™ Precise WTA Single Cell Kit for RNA 65

Preparing the Precise WTA Single Cell Encoding Plate 66
Adding RNA to the Precise WTA Single Cell Encoding Plate 66
Performing Precise reverse transcription 67

1

Introduction

About this guide

Introduction

This guide provides detailed instructions on whole transcriptome amplification (WTA) and preparation of single cell libraries with the BD™ Precise WTA Single Cell Kit using Molecular Indexing™ technology in a 96-well plate. The guide also contains instructions for preparing libraries from RNA.

Safety symbols

Introduction

This topic describes the safety symbols used in this guide.

Safety symbols

The following table lists the safety symbols used in this guide to alert you to potential hazards.

Symbol	Meaning
	Caution! Indicates the need for the user to consult the instructions for use for important cautionary information such as warnings and precautions that cannot, for a variety of reasons, be presented on the medical device itself.
	Biological hazard

Safety data sheets

Introduction

This topic describes how to obtain safety data sheets (SDS).

Obtaining SDSs

Before handling chemicals, read and understand the SDSs. To obtain SDSs for chemicals ordered from BD Genomics, contact BD Genomics technical support at techsupport.genomics@bd.com.

2

Getting Started

WTA Library preparation overview

Introduction

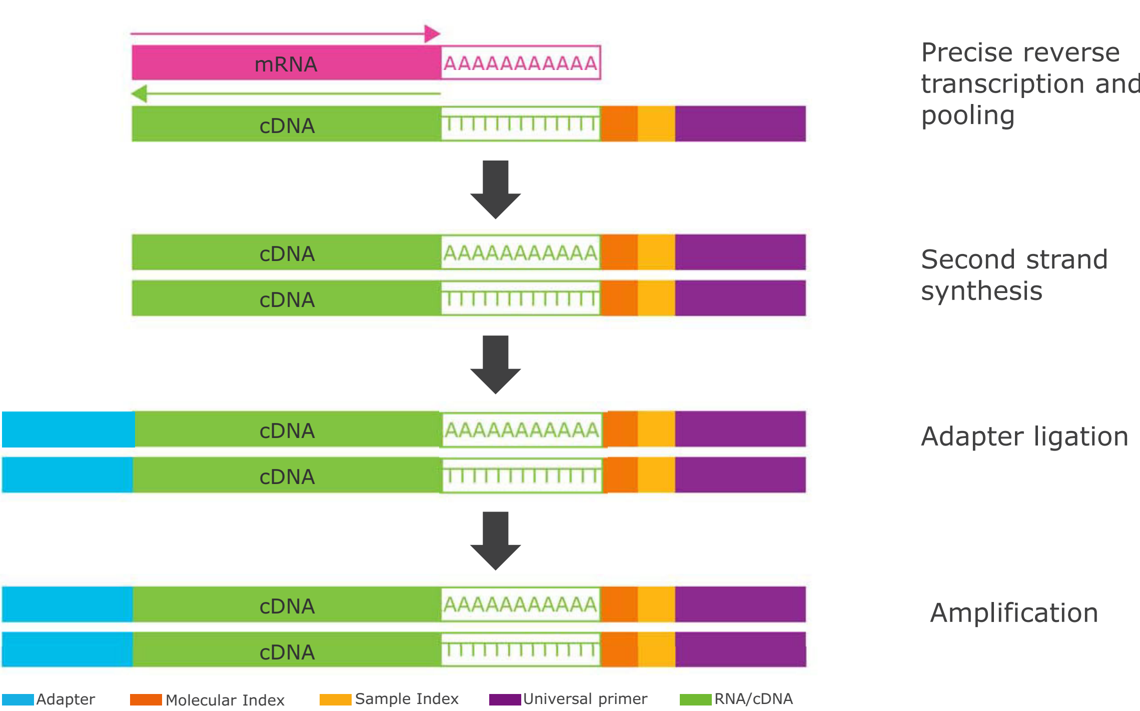
The BD™ Precise WTA Single Cell Kit is a single-cell and low-input RNA quantification kit for next-generation sequencing (NGS) analysis that is uniquely designed to work directly with flow cytometers. Using BD Molecular Indexing™, the Precise kit labels each transcript with a short DNA sequence to enable counting of mRNA. Combining phenotypic data for each cell, identified during cell sorting, and transcriptomic data for each cell enabled by patented Precise technology, results in tracking of individual cell data from isolation through data analysis for breakthrough results.

Workflow

Precise Single Cell WTA assays are in 96-well plates. Sort cells one cell per well in the plate, and then use the BD™ Precise WTA Single Cell Kit and basic molecular biology tools to prepare a sequence-ready library for Illumina sequencers.

Stage	Description	Preparation time (min)	Thermal cycler time (min)
1	Prepare the Precise WTA Single Cell Encoding Plate.	20	5
2	Perform reverse transcription, pool and purify the product.	20	40
3	Perform second strand synthesis and purify the product.	10	150
4	Ligate the cDNA to the Precise WTA Adapter and purify the product.	10	30
5	Amplify the ligation products and purify the product.	10	120
6	Perform quality control on the WTA product.	20	—
7	Perform random primer extension.	20	60
8	Amplify the primer extension product and purify the amplified library.	20	20
9	Perform quality control on the amplified library.	20	—

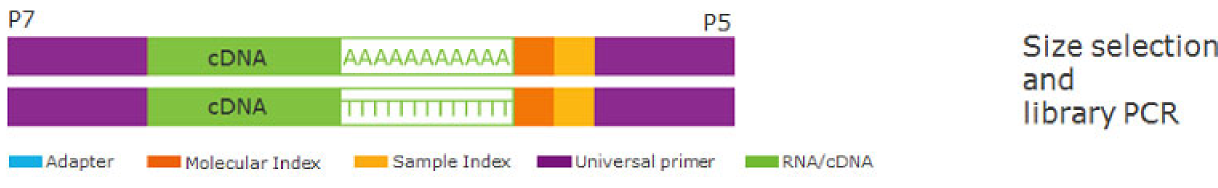
Whole transcriptome amplification preparation



cDNA AAAAAAAAAAA
cDNA TTTTTTTTTTTTT
WTA product

cDNA AAAAAAAAAA
cDNA TTTTTTTTTTTT
Denature DNA

cDNA AAAAAAAAAA
cDNA TTTTTTTTTTTT
Random primer extension



Site requirements

Workspace designation

You must dedicate two isolated workspaces in the laboratory to run high sensitivity, single-cell sequencing experiments:

- Pre-amplification workspace
- Post-amplification workspace

Sample requirements and cell sorting recommendations

Sample requirements

The BD™ Precise WTA Single Cell Kit is designed for use with sorted single cells or 10–50 pg of purified RNA per well. The cells introduced to each plate must be sorted directly into the Precise WTA Single Cell Encoding Plate.

Cell sorting recommendations

- Use a cell sorter to deliver one cell per well into the Precise WTA Single Cell Encoding Plate. Consult the manual of the cell sorter manufacturer.
- Seal the Precise WTA Single Cell Encoding Plate containing cells using clear adhesive film, vortex the plate at low speed for 5–10 seconds to mix the reagents and cells, and then centrifuge the plate briefly at 1,000 x *g* for 10 seconds.
- Snap-freeze the plate with cells. BD recommends placing the plate on a pre-chilled 96-well aluminum cooler block at –80°C.
- The centrifuged Precise WTA Single Cell Encoding Plate can be stored at –80°C for ≤6 weeks.

Thermal Cycler Programs Setup

Recommendations

- Use a properly calibrated thermal cycler for 0.2 mL tubes with a maximum reaction volume of 50 µL.
- Use a heated lid set to 100–105°C.
- For specific instrument operation, follow the instructions provided by the manufacturer.

Thermal cycler programs

Pre-program pre- and post-amplification thermal cyclers with these programs:

Program	Workspace	Library preparation step	Steps	Cycles	Temperature	Time
Program 1	Pre-amplification	Secondary structure denaturation	1	1	65°C	3 min
			2	1	4°C	∞
Program 2	Pre-amplification	Reverse transcription	1	1	42°C	30 min
			2	1	80°C	5 min
			3	1	4°C	∞
Program 3	Pre-amplification	Second strand synthesis	1	1	16°C	150 min
			2	1	4°C	∞
Program 4	Pre-amplification	Adapter ligation	1	1	23°C	30 min
			2	1	4°C	∞

Program	Workspace	Library preparation step	Steps	Cycles	Temperature	Time
Program 5	Post-amplification	WTA amplification	1	1	98°C	30 s
			2	18[a]	98°C	10 s
					58°C	15 s
					72°C	3 min
			3	1	72°C	5 min
			4	1	4°C	∞
Program 6	Post-amplification	WTA denaturation	1	1	95°C	2 min
			2	1	4°C	5 min
			3	1	4°C	∞
Program 7	Post-amplification	WTA primer extension	1	1	37°C	30 min
			2	1	80°C	20 min
			3	1	4°C	∞
Program 8	Post-amplification	Library amplification	1	1	98°C	30 s
			2	12	98°C	10 s
					65°C	15 s
					72°C	20 s
			3	1	72°C	2 min
			4	1	4°C	∞

a. Thermal cycling for 18 cycles is sufficient for most cells with the typical amount of RNA content. For cells with a low amount of RNA, like quiescent T cells and other immune cells, you may need to thermal cycle the ligation products for an additional 3 cycles. To amplify the ligation products with additional cycles, see Low signal (page 62).

Best practices

Tips for use of the BD™ Precise WTA Single Cell Kit

- Transfer all liquid product from wells and tubes, even if it means transferring a small number of beads.
- Avoid introducing bubbles while pipetting. Centrifuge samples to reduce bubbles, and then collect the liquid. Pipet liquids carefully.
- Use low-retention, RNase-free, filter pipette tips and low-binding reaction tubes to prevent absorption to plastic surfaces.

Good laboratory practices

- Calibrate pipettes every six months to ensure accurate sample volume transfer at each step.
- Avoid pipetting small volumes to maintain accuracy. Prepare master mixes as directed.
- Avoid multiple freeze/thaw cycles for samples.
- Unless otherwise specified, thaw reagents on ice. Store reagents at their specified storage conditions.
- Collect small volumes by briefly centrifuging samples. Brief or pulse centrifugation is <1 second.
- Work in designated pre- or post-amplification workspaces according to the protocol.

RNase-free technique

Prevent the introduction of endogenous RNases into samples during processing by:

- Wear disposable gloves and change them frequently.
- Never reuse tips or tubes.
- Keep tip boxes, reagent containers and sample tubes closed when not in use.
- Always maintain a clean laboratory bench and if necessary, wipe work surface with a solution of 10% bleach.

3

Product information

Kits and storage conditions

Introduction

The BD™ Precise WTA Single Cell Kit includes two 96-well Precise WTA Single Cell Encoding Plates and reagents for the preparation of two libraries from up to 192 cells or samples.

Storage conditions

Unless otherwise specified, thaw reagents on ice. Store reagents at their specified storage conditions.

Use only non-frost free freezers for all reagent storage.

Product name	Component	Quantity (vial)	Volume (µL)	Storage
BD™ Precise WTA Single Cell Kit—2 pack (BD Genomics PN 634100)	Precise WTA Single Cell Encoding Plate	2 plates	—	–20°C
	Precise RT Buffer	1	600	
	Precise RNase Inhibitor	1	35	
	Precise Reverse Transcriptase	1	60	
	Precise SS Enzyme Mix	1	20	
	Precise SS Synthesis Buffer	1	100	
	Precise SS Stop Solution	1	20	
	Precise Ligase	1	15	
	Precise Ligase Buffer	1	40	
	Precise WTA Adapter	1	20	
	Precise WTA PCR Mastermix	1	360	
	Precise WTA PCR Primer	1	50	
	Precise Elution Buffer	2	500	

Product name	Component	Quantity (vial)	Volume (µL)	Storage
	Nuclease-Free Water	1	1,100	–20°C
	Precise Reaction Buffer	1	30	
	Precise Extension Enzyme	1	20	
	Precise WTA Library Random Primer Mix	1	30	
	Precise WTA Library Forward Primer	1	20	
	Precise WTA Library Index Primer 1	1	20	
	Precise WTA Library Index Primer 2	1	20	

Suggested kits from other vendors

Reagent	Supplier	Part no.
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Agilent DNA High Sensitivity Kit	Agilent Technologies	5067-4626

Reagents

Required reagents

Reagent	Supplier	Part no.
Agencourt AMPure XP magnetic beads	Beckman Coulter	A63880
Ethyl Alcohol, Pure	Sigma-Aldrich	E7023-500ML
Bleach	Major supplier (MS)	—

Consumables

Required consumables

Consumable item	Supplier	Part no.
Eppendorf® LoBind microcentrifuge tubes, 1.5 mL	Sigma-Aldrich	Z666548-250
Eppendorf® LoBind microcentrifuge tubes, 2.0 mL	Sigma-Aldrich	Z666556-250
GeneMate SnapStrip® 8-Strip Standard PCR Tubes with Individually Attached Flat Caps	Genemate	T-3196-1
MicroAmp® Clear Adhesive Film (plate seals)	Thermo Fisher Scientific	4306311
RNase-free filter pipette tips	MS	—
Disposable gloves	MS	—

Suggested consumables

Consumable item	Supplier	Part no.
Qubit Assay Tubes	Thermo Fisher Scientific	Q32856

Equipment

Required equipment

Supply pre- and post-amplification workspaces with the required equipment. You may need two sets of some equipment.

Equipment item	Supplier	Part no.
6-Tube Magnetic Separation Rack	New England Biolabs	S1506S
Low Profile Magnetic Separation Stand for 0.2 mL, 8-strip tubes[a]	V&P Scientific, Inc.	VP772F4-1
Pipettes (P2, P10, P20, P200, P1000)	Major supplier (MS)	—
12-channel pipette (2–10 µL)	MS	—
Microcentrifuge for 1.5–2.0 mL tubes	MS	—
Microcentrifuge for 0.2 mL tubes	MS	—
Centrifuge for 96-well plates	MS	—
Vortexer	MS	—
Thermal cycler with heated lid	MS	—
96-well aluminum cooler block	MS	—

a. An alternative is the Magnetic Separator–PCR Strip (Clontech PN 635011).

Suggested equipment

Equipment item	Supplier	Part no.
2100 Bioanalyzer® or equivalent	Agilent	G2940CA
Qubit 3.0 Fluorometer or equivalent	Thermo Fisher Scientific	Q33216

4

Preparing a single cell library with the BD™ Precise WTA Single Cell Kit

Preparing the Precise WTA Single Cell Encoding Plate

Before you begin

Note: To prepare the BD Precise WTA library from RNA, see Preparing a library with the BD™ Precise WTA Single Cell Kit for RNA (page 65).

Prepare the pre-amplification workspace for preparation of the Precise WTA Single Cell Encoding Plate.

Procedure

1. Centrifuge the Precise WTA Single Cell Encoding Plate (PN 700000020) at 1,000 x *g* for 10 seconds to collect the 5 µL of reagents at the bottom of each well.
2. Place the plate on ice in a 96-well aluminum cooler block and slowly peel the seal from the plate to avoid contamination between wells. If there is evidence of cross-contamination, dispose of the plate and use a new plate.
3. Sort cells one cell per well in the plate. BD recommends leaving up to four wells empty for non-template controls. See Cell sorting recommendations (page 14).
4. Seal the plate using clear adhesive film, and, if necessary, store the plate.

Performing BD Precise reverse transcription

Before you begin

Prepare the pre-amplification workspace for reverse transcription (RT) of the samples.

Denaturing the samples

Note: The reagents in the Precise WTA Single Cell Encoding Plate will thaw during centrifugation.

1. Centrifuge the sealed Precise WTA Single Cell Encoding Plate at centrifuge 1,000 x *g* for 10 seconds to collect the samples in the wells. Place the plate in a 96-well ice-cooled rack.
2. Run **program 1** on the samples in the pre-amplification thermal cycler:

Step	Cycles	Temperature	Time
RNA secondary structure denaturation	1	65°C	3 min
Hold	1	4°C	∞

3. Centrifuge the plate at 1,000 x *g* for 10 seconds, and then place it in a 96-well ice-cooled rack.

Preparing the RT enzyme master mix

1. Place a 0.2 mL 12-tube strip on ice.
2. Thaw the Precise RT Buffer (PN 650000039) at room temperature and keep enzymes on ice.
3. In the pre-amplification workspace, in a new 1.5 mL LoBind tube, prepare the RT enzyme master mix by adding the components in this order:

Component	Volume per well (µL)	Volume for 96-well plate with overage (µL)
Nuclease-Free Water (PN 650000052)	2.7	324.0
Precise RT Buffer (PN 650000039)	2.0	240.0
Precise RNase Inhibitor (PN 650000040)	0.1	12.0
Precise Reverse Transcriptase (PN 650000041)	0.2	24.0
Total	**5.0**	**600.0**

4. Briefly vortex and centrifuge the mix.
5. Pipet 50 µL of the RT enzyme master mix into each well of the strip tube that is on ice.
6. Slowly peel the seal from the Precise WTA Single Cell Encoding Plate to avoid contamination between wells.

7. Using new tips after each pipetting, use a 12-channel pipette to dispense 5.0 µL of the RT enzyme master mix into each of the 96 wells. The final RT reaction volume in each well is 10.0 µL.

8. Seal the plate with clear adhesive film, vortex at low speed for 10 seconds to mix the samples, and then centrifuge the plate at 1,000 x g for 10 seconds.

Performing reverse transcription

1. Run thermal cycler **program 2** on the samples in the pre-amplification thermal cycler:

Step	Cycles	Temperature	Time
Reverse transcription	1	42°C	30 min
Heat inactivation	1	80°C	5 min
Hold	1	4°C	∞

2. Remove the samples ≤60 minutes after thermal cycling.

3. Centrifuge the plate at 1,000 x g for 10 seconds, and then place it on ice.

Pooling the samples

Before you begin

Prepare the pre-amplification workspace for sample pooling.

The Precise WTA Single Cell Encoding Plate contains detergent. Avoid introducing bubbles while pipetting.

Procedure

1. Bring the reverse-transcribed samples to room temperature.
2. Use a 12-channel pipette to transfer the samples from the top seven rows (A–G) of the plate into the last row (row H).
3. Use a single-channel pipette to combine the 12 reactions into a new 2 mL tube. The final volume is 900 µL±50 µL.
4. If necessary, to reduce bubbles, briefly centrifuge the combined reactions (<1 second).

Purifying the pooled samples

About this procedure

This procedure includes detailed instructions on reagent preparation and purification of the pooled samples with Agencourt® AMPure® XP magnetic beads.

Before you begin

- Prepare the pre-amplification workspace for the purification.
- Bring the Agencourt® AMPure® XP magnetic beads to room temperature.
- Review Tips for use of the BD™ Precise WTA Single Cell Kit (page 17).

Purifying the pooled samples

1. In a new 15 mL conical tube, prepare 80% (v/v) ethyl alcohol by pipetting 2.0 mL of nuclease-free water and adding absolute ethyl alcohol to 10.0 mL. Cap and vortex the tube for 10 seconds.

   Prepare 80% ethyl alcohol fresh and use ≤1 day. Prepare separate solutions for pre- and post-amplifications workspaces. Do not interchange solutions.

2. Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
3. Pipet 900 µL of beads to the 2 mL tube containing the pooled samples.
4. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample (<1 second).
5. Incubate the suspension at room temperature for 5 minutes, and then place it on the magnet for 5 minutes until the solution is clear.
6. Without disturbing the beads and leaving the tube on the magnet, carefully remove and discard only the supernatant. Avoid withdrawing beads from the top of the supernatant.
7. Keeping the tube on the magnet, gently pipet 2 mL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. You can leave the tube open.
8. Incubate the sample at room temperature for 30 seconds on the magnet.
9. Without disturbing the beads and leaving the tube on the tube magnet, carefully remove and discard the supernatant.
10. Repeat steps 7–9 once for a total of two washes.
11. Leaving the tube on the magnet, gently tap the stand (~2–3 times) until you collect the liquid at the bottom of the tube.
12. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

Drying the beads and eluting the sample

1. Leave the tube open on the magnet to air dry the beads at room temperature for 3 minutes.
2. Pipet 72 µL of Precise Elution Buffer (PN 650000051) into the tube, close the tube, and then vortex to resuspend the beads.
3. Briefly centrifuge (<1 second) the tube to collect contents at the bottom of the tube, and then incubate the sample at room temperature for 2 minutes.
4. Place the tube on the magnet until the solution is clear, usually in ≤30 seconds.
5. Pipet the eluate (~72 µL) to a new 0.2 mL PCR tube that is on ice.
6. Proceed immediately to Performing second strand (SS) synthesis on the pooled samples (page 35).

Performing second strand (SS) synthesis on the pooled samples

About this procedure

Use the diluted second strand enzyme mix to perform second strand synthesis on the pooled samples. Purify the second strand synthesis products using Agencourt AMPure XP magnetic beads.

Before you begin

- Prepare the pre-amplification workspace for second strand synthesis on the pooled samples.
- Keep the pooled samples and reagents on ice.

Preparing the second strand synthesis mix

Mix enzymes by pipette only.

1. To a 1.5 mL LoBind tube, add the following components in this order to prepare the diluted Precise SS Enzyme Mix:

Component	Volume (µL)
Precise SS Synthesis Buffer (PN 650000044)	38.0
Precise SS Enzyme Mix (PN 650000043)	2.0
Total	**40.0**

2. Gently mix the diluted Precise SS Enzyme Mix by pipette only. Briefly centrifuge the tube, and then place it on ice.

3. In the 0.2 mL PCR tube containing the purified samples, add the diluted Precise SS Enzyme Mix to prepare the second strand synthesis mix:

Component	Volume (µL)
Purified pooled samples [See Purifying the pooled samples (page 32).]	72.0
Diluted Precise SS Enzyme Mix (See step 1.)	8.0
Total	**80.0**

4. Gently mix the second strand synthesis mix by pipette only, and briefly centrifuge the tube.
5. Keep the sample on ice until the thermal cycler is at 16°C.

Performing second strand synthesis

1. Run thermal cycler **program 3** on the SS synthesis master mix in the pre-amplification thermal cycler:

Step	Cycles	Temperature	Time
Incubation	1	16°C	150 min
Hold	1	4°C	∞

2. Remove the samples ≤60 minutes after thermal cycling.
3. Immediately put the second strand synthesis products on ice, and keeping them on ice, add 5 µL of Precise SS Stop Solution (PN 650000045) to the products. The total volume is 85 µL.
4. Vortex and briefly centrifuge the products, and then place them at room temperature.

Purifying the second strand synthesis products

Before you begin

- Prepare the pre-amplification workspace for the purification.
- Obtain fresh 80% ethyl alcohol. See Purifying the pooled samples (page 33).
- Bring the Agencourt AMPure XP magnetic beads to room temperature.
- Review Tips for use of the BD™ Precise WTA Single Cell Kit (page 17).

Purifying the second strand synthesis products

1. Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Pipet 100 µL of beads to the single tube containing the 85 µL second strand synthesis products.
3. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample (<1 second).
4. Incubate the suspension at room temperature for 5 minutes, and then place it on the magnet for 5 minutes until the solution is clear.
5. Without disturbing the beads and leaving the tube on the magnet, carefully remove and discard only the supernatant. Avoid withdrawing beads from the top of the supernatant.
6. Keeping the tube on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. You can leave the tube open.
7. Incubate the sample at room temperature for 30 seconds on the magnet.
8. Without disturbing the beads and leaving the tube on the tube magnet, carefully remove and discard the supernatant.
9. Repeat steps 6–8 once for a total of two washes.

10. Leaving the tube on the magnet, gently tap the stand (~2–3 times) until you collect the liquid at the bottom of the tube.
11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

Drying the beads and eluting the sample

1. Leave the tube on the magnet to air dry the beads at room temperature for 3 minutes.
2. Pipet 55 µL of Precise Elution Buffer (PN 650000051) into the tube, close the tube, and then vortex to resuspend the beads.
3. Briefly centrifuge (<1 second) the tube to collect contents at the bottom of the tube, and then incubate the sample at room temperature for 2 minutes.
4. Place the tube on the magnet until the solution is clear, usually in ≤30 seconds.
5. Pipet the eluate (~55 µL) to a new 0.2 mL PCR tube that is on ice.
6. Proceed immediately to Ligating the Precise WTA Adapter to the second strand synthesis products (page 39).

Ligating the Precise WTA Adapter to the second strand synthesis products

About this procedure

Prepare the cDNA ligation master mix with the purified second strand synthesis products. Next, prepare the cDNA ligation mix. Run program 4 to ligate the Precise WTA Adapter to the second strand synthesis products and purify the ligation products using Agencourt AMPure XP magnetic beads.

Before you begin

Prepare the pre-amplification workspace for the ligation.

Preparing the cDNA ligation master mix and cDNA ligation mix

Precise Ligase Buffer is viscous. Slowly pipet it using low-bind pipette tips.

Mix enzymes by pipette only.

1. In the 0.2 mL PCR tube containing 55 µL of the second strand synthesis products, add 15 µL of Precise Ligase Buffer (PN 650000047) for a total volume of 70.0 µL. This is the cDNA ligation master mix.
2. Vortex and briefly centrifuge the mix, and then place it at room temperature.

3. In the 0.2 mL tube containing the cDNA ligation master mix, add the following components in this order to prepare the cDNA ligation mix:

Component	Volume (µL)
cDNA ligation master mix (See step 1.)	70.0
Precise WTA Adapter (PN 650000048)	3.0
Precise Ligase (PN 650000046)	5.0
Total	**78.0**

4. Gently mix the cDNA ligation mix by pipette only, and briefly centrifuge the tube.

Performing ligation

1. Run thermal cycler **program 4** on the cDNA ligation master mix in the pre-amplification thermal cycler:

Step	Cycles	Temperature	Time
Incubation	1	23°C	30 min
Hold	1	4°C	∞

2. Briefly centrifuge the products, and then place them at room temperature.

Purifying the ligation products

Before you begin

- Prepare the pre-amplification workspace for the purification.
- Obtain fresh 80% ethyl alcohol. See Purifying the pooled samples (page 33).
- Bring the Agencourt AMPure XP magnetic beads to room temperature.
- Review Tips for use of the BD™ Precise WTA Single Cell Kit (page 17).

Purifying the ligation products

1. Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Pipet 75 µL of beads to the single tube containing the 78 µL of ligation products.
3. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample (<1 second).
4. Incubate the suspension at room temperature for 5 minutes, and then place it on the magnet for 5 minutes until the solution is clear.
5. Without disturbing the beads and leaving the tube on the magnet, carefully remove and discard only the supernatant. Avoid withdrawing beads from the top of the supernatant.
6. Keeping the tube on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. You can leave the tube open.
7. Incubate the sample at room temperature for 30 seconds on the magnet.
8. Without disturbing the beads and leaving the tube on the tube magnet, carefully remove and discard the supernatant.
9. Repeat steps 6–8 once for a total of two washes.

10. Leaving the tube on the magnet, gently tap the stand (~2–3 times) until you collect the liquid at the bottom of the tube.
11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

Drying the beads and eluting the sample

1. Leave the tube open on the magnet to air dry the beads at room temperature for 3 minutes.
2. Pipet 55 µL of Precise Elution Buffer (PN 650000051) into the tube, close the tube, and then vortex to resuspend the beads.
3. Briefly centrifuge (<1 second) the tube to collect contents at the bottom of the tube, and then incubate the sample at room temperature for 2 minutes.
4. Place the tube on the magnet until the solution is clear, usually in ≤30 seconds.
5. Place four new 0.2 mL PCR tubes or a strip of 0.2 mL tubes on ice. You will split the master mix into four reactions in a later step.
6. Pipet the eluate (~55 µL) to one of the 0.2 mL PCR tubes that is on ice.
7. Proceed immediately to Amplifying the ligation products (page 43).

Amplifying the ligation products

Before you begin

- Prepare the *pre*-amplification workspace for preparation of the PCR master mix.
- Prepare the *post*-amplification workspace for library amplification.

Preparing the PCR master mix

1. In the pre-amplification workspace, in the 0.2 mL tube containing the purified ligation products, add the following components in this order to prepare PCR master mix:

   Note: If there is precipitate in the Precise WTA PCR Mastermix, vortex the mix until the precipitate disappears. If the precipitate does not disappear, contact BD Genomics technical support at techsupport.genomics@bd.com.

Component	Volume (µL)
Ligation products [See Purifying the ligation products (page 41).]	55.0
Nuclease-Free Water (PN 650000052)	25.0
Precise WTA PCR Primer (PN 650000050)	20.0
Precise WTA PCR Mastermix (PN 650000049)	100.0
Total	**200.0**

2. Gently mix the PCR master mix by pipette only. Briefly centrifuge the tube, and then place it on ice.
3. Pipet 50 µL of the PCR master mix into each of the three new 0.2 mL PCR tubes on ice. You have a total of four tubes with 50 µL of PCR master mix each.
4. Briefly centrifuge the tubes.

Amplifying the ligation products

If the RNA content in the cells is low (<<10 pg RNA/cell), amplify the ligation products with 20 cycles.

1. In the post-amplification workspace, run thermal cycler **program 5** on the PCR master mix in the post-amplification thermal cycler:

Step	Cycles	Temperature	Time
Hot start	1	98°C	30 s
Denaturation	18[a]	98°C	10 s
Annealing		58°C	15 s
Extension		72°C	3 min
Final extension	1	72°C	5 min
Hold	1	4°C	∞

a. Thermal cycling for 18 cycles is sufficient for most cells with the typical amount of RNA content. For cells with a low amount of RNA, like quiescent T cells and other immune cells, you may need to thermal cycle the ligation products for an additional 3 cycles. To amplify the ligation products with additional cycles, see Low signal (page 62).

Stopping point: The PCR can run overnight.

2. In a post-amplification workspace, combine the four PCR reactions into a new 1.5 mL LoBind tube. This is the WTA product.

Purifying the WTA product

Before you begin

- Prepare the *post*-amplification workspace for the purification.
- Obtain fresh 80% ethyl alcohol. See Purifying the pooled samples (page 33).
- Bring the Agencourt AMPure XP magnetic beads to room temperature.
- Review Tips for use of the BD™ Precise WTA Single Cell Kit (page 17).

Purifying the WTA product

1. Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Pipet 200 µL of beads to the tube containing the 200 µL of the WTA product.
3. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample.
4. Incubate the suspension at room temperature for 5 minutes, and then place it on the magnet for 5 minutes until the solution is clear.
5. Without disturbing the beads and leaving the tube on the magnet, carefully remove and discard only the supernatant. Avoid withdrawing beads from the top of the supernatant.
6. Keeping the tube on the magnet, gently pipet 1 mL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. You can leave the tube open.
7. Incubate the sample at room temperature for 30 seconds on the magnet.
8. Without disturbing the beads and leaving the tube on the tube magnet, carefully remove and discard the supernatant.
9. Repeat steps 6–8 once for a total of two washes.

10. Leaving the tube on the magnet, gently tap the stand (~2–3 times) until you collect the liquid at the bottom of the tube.
11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

Drying the beads and eluting the sample

1. Leave the tube open on the magnet to air dry the beads at room temperature for 3 minutes.
2. Pipet 20 µL of Precise Elution Buffer (PN 650000051) into the tube, close the tube, and then vortex to resuspend the beads.
3. Briefly centrifuge (<1 second) the tube to collect contents at the bottom of the tube, and then incubate the sample at room temperature for 2 minutes.
4. Place the tube on the magnet until the solution is clear, usually in ≤30 seconds.
5. Pipet the eluate (~20 µL) to a new 1.5 mL LoBind tube.

Do not return to the pre-amplification workspace with the amplified library.

Stopping point: The purified WTA product can be stored at –20°C for ≤6 months.

Performing quality control (QC) on the WTA product

Before you begin

Perform quality control in a post-amplification workspace.

Measuring the concentration of the WTA product

1. Use one of these methods to quantify the WTA product, in this order of preference: Qubit dsDNA HS Assay Kit > Picogreen Fluorescent Assays > Agilent Bioanalyzer®. Use 2 µL of the WTA product with Qubit. Follow the manufacturer’s instructions.

2. If the estimated concentration of the WTA product is:

   – ≥5 ng/µL, proceed to Verifying the WTA product size and purity.

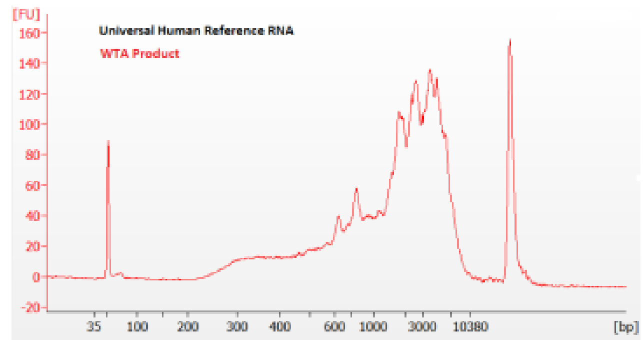
   – <5 ng/µL, proceed to Low signal (page 62).

Verifying the WTA product size and purity

1. Dilute the WTA product to ~1 ng/µL.

2. Load 1 µL of the diluted WTA product into a High Sensitivity DNA Chip and run the chip on the Agilent Bioanalyzer. Run the Agilent Bioanalyzer according to the manufacturer’s instructions.

   Typical Agilent Bioanalyzer data:



3. If the WTA product passes QC, proceed to Performing random primer extension. If the WTA product does not pass QC, contact techsupport.genomics@bd.com.

Performing random primer extension

Before you begin

- Prepare the *pre*-amplification workspace for preparation of the primer extension master mix.
- Prepare the *post*-amplification workspace for denaturing and priming the WTA product.

Preparing the priming master mix

1. From the estimated concentration [see Measuring the concentration of the WTA product (page 47)], calculate the volume of WTA product needed to add 50 ng to the reaction.
2. In the post-amplification workspace, in a new 0.2 mL PCR tube, add the following components in this order to prepare the priming master mix:

Component	Volume (µL)
Nuclease-Free Water (PN 650000052)	$34.0 - x$
Precise WTA Library Random Primer Mix (PN 650000056)	10.0
WTA product, 50 ng (See step 1.)	x
Total	**44.0**

3. Gently mix the priming master mix by pipette only, and briefly centrifuge the tube.

Denaturing and priming the WTA product

1. Run thermal cycler **program 6** on the priming master mix in the post-amplification thermal cycler:

Step	Cycles	Temperature	Time
Denaturation	1	95°C	2 min
Hold	1	4°C	5 min
Hold	1	4°C	∞

2. Place the denatured WTA product on ice.

   Note: Use the priming master mix to prepare the primer extension reaction mix.

Preparing the primer extension reaction mix

1. In the pre-amplification workspace, in a new 1.5 mL LoBind tube, add the following components in this order to prepare the primer extension master mix:

Component	Volume (µL)
Precise Reaction Buffer (PN 650000053)	7.5
Precise Extension Enzyme (PN 650000055)	1.5
Total	**9.0**

2. Gently mix the primer extension master mix by pipette only. Briefly centrifuge the tube, and then place it on ice.

3. In the post-amplification workspace, in the 0.2 mL PCR tube containing the 44.0µL of the denatured WTA product [see Denaturing and priming the WTA product (page 49)], add 6.0µL of the primer extension master mix (see step 1) for a total of 50.0µL. This is the primer extension reaction mix.

4. Gently mix the primer extension reaction mix by pipette only, briefly centrifuge the tube, and then place on ice.

Primer extension of the WTA product

Run thermal cycler **program 7** on the primer extension reaction mix in the post-amplification thermal cycler:

Step	Cycles	Temperature	Time
Incubation	1	37°C	30 min
Heat inactivation	1	80°C	20 min
Hold	1	4°C	∞

Stopping point: The PCR can run overnight.

Purifying the primer extension product

Before you begin

- Prepare the post-amplification workspace for the purification.
- Obtain fresh 80% ethyl alcohol. See Purifying the pooled samples (page 33).
- Bring the Agencourt AMPure XP magnetic beads to room temperature.
- Review Tips for use of the BD™ Precise WTA Single Cell Kit (page 17).

Purifying the primer extension product

1. Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Pipet 35 µL of beads to the tube containing the 50 µL of the primer extension product.
3. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample.
4. Incubate the suspension at room temperature for 5 minutes, and then place it on the magnet for 5 minutes until the solution is clear.
5. Without disturbing the beads and leaving the tube on the magnet, carefully remove and discard only the supernatant. Avoid withdrawing beads from the top of the supernatant.
6. Keeping the tube on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. You can leave the tube open.
7. Incubate the sample at room temperature for 30 seconds on the magnet.
8. Without disturbing the beads and leaving the tube on the tube magnet, carefully remove and discard the supernatant.
9. Repeat steps 6–8 once for a total of two washes.

10. Leaving the tube on the magnet, gently tap the stand (~2–3 times) until you collect the liquid at the bottom of the tube.
11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

Drying the beads and eluting the sample

1. Leave the tube open on the magnet to air dry the beads at room temperature for 3 minutes.
2. Pipet 20 µL of Precise Elution Buffer (PN 650000051) into the tube, close the tube, and then vortex to resuspend the beads.
3. Briefly centrifuge (<1 second) the tube to collect contents at the bottom of the tube, and then incubate the sample at room temperature for 2 minutes.
4. Place the tube on the magnet until the solution is clear, usually in ≤30 seconds.
5. Pipet the eluate (~20 µL) to a new 0.2 mL PCR tube that is on ice.

Do not return to the pre-amplification workspace with the primer extension product.

Amplifying the primer extension product

Before you begin

- Prepare the *pre*-amplification workspace for preparation of the library amplification master mix and amplification mix.
- Prepare the *post*-amplification workspace for amplification of the library.

Preparing the library amplification master mix

1. In the pre-amplification workspace, in a new 0.2 mL PCR tube, add the following components in this order to prepare the library amplification master mix:

Component	Volume (µL)
Precise WTA PCR Mastermix (PN 650000049)	25.0
Precise WTA Library Forward Primer (PN 650000057)	2.5
Precise WTA Library Index Primer 1 (PN 650000058) or Precise WTA Library Index Primer 2 (PN 650000059) [a]	2.5
Total	**30.0**

a. Use Precise WTA Library Index Primers 1 or 2 to multiplex multiple libraries in a single sequencing run.

2. Gently mix the library amplification master mix by pipette only, and briefly centrifuge the tube.

Preparing the amplification mix

1. In the post-amplification workspace, in the 0.2 mL PCR tube containing the 30.0 µL of the library amplification master mix [see Preparing the library amplification master mix (page 53)], add the 20.0 µL of the primer extension product [See Purifying the primer extension product (page 51)] for a total of 50.0µL.
2. Gently mix the amplification mix by pipette only, and briefly centrifuge the tube.

Amplifying the amplification mix

Run thermal cycler **program 8** on the PCR master mix in the post-amplification thermal cycler:

Step	Cycles	Temperature	Time
Hot start	1	98°C	30 s
Denaturation	12	98°C	10 s
Annealing		65°C	15 s
Extension		72°C	20 s
Final extension	1	72°C	2 min
Hold	1	4°C	∞

Stopping point: The PCR can run overnight.

Purifying the amplified library

Before you begin

- Prepare the post-amplification workspace for the purification.
- Obtain fresh 80% ethyl alcohol. See Purifying the pooled samples (page 33).
- Bring the Agencourt AMPure XP magnetic beads to room temperature.
- Review Tips for use of the BD™ Precise WTA Single Cell Kit (page 17).

Purifying the amplified library

1. Vortex the Agencourt AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Pipet 35 µL of beads to the tube containing the 50 µL of the amplified library.
3. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample.
4. Incubate the suspension at room temperature for 5 minutes, and then place it on the magnet for 5 minutes until the solution is clear.
5. Without disturbing the beads and leaving the tube on the magnet, carefully remove and discard only the supernatant. Avoid withdrawing beads from the top of the supernatant.
6. Keeping the tube on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. You can leave the tube open.
7. Incubate the sample at room temperature for 30 seconds on the magnet.
8. Without disturbing the beads and leaving the tube on the tube magnet, carefully remove and discard the supernatant.
9. Repeat steps 6–8 once for a total of two washes.

10. Leaving the tube on the magnet, gently tap the stand (~2–3 times) until you collect the liquid at the bottom of the tube.
11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

Drying the beads and eluting the sample

1. Leave the tube open on the magnet to air dry the beads at room temperature for 3 minutes.
2. Pipet 20 µL of Precise Elution Buffer (PN 650000051) into the tube, close the tube, and then vortex to resuspend the beads.
3. Briefly centrifuge (<1 second) the tube to collect contents at the bottom of the tube, and then incubate the sample at room temperature for 2 minutes.
4. Place the tube on the magnet until the solution is clear, usually in ≤30 seconds.
5. Pipet the eluate (~20 µL) into a new tube.

Do not return to the pre-amplification workspace with the amplified library.

Stopping point: The purified and amplified library can be stored at –20°C for ≤6 months.

Performing quality control on the amplified library

Before you begin

Prepare the post-amplification workspace for quality control.

Measuring the concentration of the amplified library

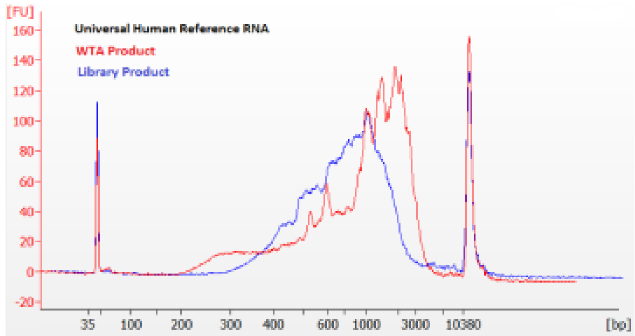
Use the Qubit dsDNA HS Assay Kit or Picogreen Fluorescent Assays to quantify the amplified library. Use 2 µL of the amplified library with Qubit and follow the manufacturer's instructions. For Picogreen assays, carefully follow the manufacturer's instructions to quantify low sample concentrations.

Library concentrations range from 10–30 ng/µL.

Verifying the amplified library size and purity

1. Dilute the amplified library to ~1–3 ng/µL.
2. Load 1 µL of the diluted amplified library into a High Sensitivity DNA Chip and run the chip on the Agilent Bioanalyzer. Run the Agilent Bioanalyzer according to the manufacturer's instructions.

   Typical Agilent Bioanalyzer data:



3. If the amplified library passes QC, sequence the library on an Illumina sequencer. Follow the Illumina guidelines for sequencing. If the library does not pass, see Troubleshooting (page 61).

Sequencing the amplified library

Introduction

By using the BD™ Precise WTA Single Cell Kit and Illumina compatible sequencing primers:

- The amplified library consists of fragments ranging from 300–1,000 bp.
- One amplified library is prepared for each 96-well plate.

If you are running plate index samples, the indexes are equivalent to Illumina indexed adapters D701 (ATTACTCG) and D702 (TCCGGAGA).

Procedure

1. Sequence the library according to these requirements:

Parameter	Requirement
Platform	Illumina
Paired-end reads	Yes
Minimum read length	75 x 75
Paired reads per cell	250,000–350,000[a]

a. A targeted range.

2. Analyze the Illumina sequence files with the Cellular Research BD™ Precise Whole Transcriptome Assay Analysis Pipeline v2.0 on the Seven Bridges Genomics platform. See the *BD™ Precise Whole Transcriptome Analysis User's Guide For Illumina Sequencing* (PN 910000017).

References

Fu, GK, Xu, W, Wilhelmy, J, Mindrinos, M, Davis, R, Xiao, W, Fodor, SPA. Molecular indexing enables quantitative targeted RNA sequencing and reveals poor efficiencies in standard library preparations. *Proc Natl Acad Sci U S A*, 2014;111:1891-96. PMID: 24449890.

Fu, GK, Wilhelmy, J, Stern, D, Fan, C, and Fodor, SPA. Digital encoding of cellular mRNAs enables precise and absolute gene expression measurement by single-molecule counting. *Analytical Chemistry*. (2014);86:2867-2870. PMID: 24579851.

Fu GK, Hu J, Wang PH, and Fodor SP. Counting individual DNA molecules by the stochastic attachment of diverse labels. *Proc Natl Acad Sci U S A*. 2011;108:9026-31. PMID: 21562209.

5

Troubleshooting

Sequencing

Introduction

This topic describes possible problems and recommended solutions for low WTA yield issues.

Low signal

Possible causes	Recommended solutions
Overdried Agencourt AMPpure beads	Dry beads the minimum time until no droplets are visible and the pellet is not shiny and before it is cracked. Leave the tube open on the magnet to air dry the beads at room temperature for 3 minutes.
Low PCR yield of WTA product (<5 ng/µL)	1. In the pre-amplification workspace, in a new 1.5 mL LoBind tube containing ligation products, add the following components in this order to prepare the PCR master mix:

Component	Volume (µL)
Low-yield WTA product [see Purifying the WTA product (page 45)]	15.0
Nuclease-Free Water (PN 650000052)	5.0
Precise WTA PCR Primer (PN 650000050)	5.0
Precise WTA PCR Mastermix (PN 650000049)	25.0
Total	**50.0**

Possible causes	Recommended solutions
Low PCR yield of WTA product (<5 ng/µL)	2. *Gently* mix the PCR master mix by pipette only, and briefly centrifuge the tube. 3. Run thermal cycler **program 5** on the PCR master mix in the *post*-amplification thermal cycler for an additional 3 cycles. The PCR can run at 4°C overnight. See Thermal cycler programs (page 15):

Step	Cycles	Temperature	Time
Hot start	1	98°C	30 s
Denaturation		98°C	10 s
Annealing	3	58°C	15 s
Extension		72°C	3 min
Final extension	1	72°C	5 min
Hold	1	4°C	∞

Possible causes	Recommended solutions
Low PCR yield of WTA product (<5 ng/µL)	4. Proceed to Purifying the WTA product (page 45). Use 50 µL of Agencourt AMPure XP magnetic beads (equal volume to re-amplified ligation products). 5. Measure the concentration and verify the size and purity of the WTA product after the additional thermal cycles. See Performing quality control (QC) on the WTA product (page 47).

A

Preparing a library with the BD™ Precise WTA Single Cell Kit for RNA

Preparing the Precise WTA Single Cell Encoding Plate

Before you begin

Note: To prepare the BD Precise WTA library from single cells, see Chapter 4: Preparing a single cell library with the BD™ Precise WTA Single Cell Kit (page 27).

Prepare the pre-amplification workspace for preparation of the Precise WTA Single Cell Encoding Plate.

Procedure

1. Centrifuge Precise WTA Single Cell Encoding Plate at 1,000 x *g* for 10 seconds to collect the 5 µL of reagents in each well.
2. Place the plate on ice in a 96-well aluminum cooler block and slowly peel the seal from the plate to avoid contamination between wells. If there is evidence of cross-contamination, appropriately dispose of the plate and use a new plate.

Adding RNA to the Precise WTA Single Cell Encoding Plate

Before you begin

Prepare the pre-amplification workspace for adding RNA to the Precise WTA Single Cell Encoding Plate.

Adding RNA to the plate

1. Ensure that each RNA sample is 10–50 pg and that there are no RNases or reverse transcriptase inhibitors in the samples. If there are RNases or RT inhibitors, remove them by using appropriate purification methods.
2. Using new tips after each pipetting, dispense 2.7 µL of RNA in nuclease-free water into each sample well and 2.7 µL of nuclease-free water into each no template control (NTC) well. Avoid introducing bubbles while pipetting.

3. Seal the plate with clear adhesive film, vortex it for 5–10 seconds to mix the reagents, and then centrifuge the plate at 1,000 x g for 10 seconds

Stopping point: You can store the plate at –80°C for ≤6 weeks.

Performing Precise reverse transcription

Before you begin

Prepare the pre-amplification workspace for reverse transcription (RT) of the samples.

Denaturing the samples

Note: The reagents in the Precise WTA Single Cell Encoding Plate thaw during centrifugation.

1. Centrifuge the sealed Precise WTA Single Cell Encoding Plate at centrifuge 1,000 x *g* for 10 seconds to collect the samples in the wells. Place the plate in a 96-well ice-cooled rack.
2. Run **program 1** in the pre-amplification thermal cycler:

Step	Cycles	Temperature	Time
RNA secondary structure denaturation	1	65°C	3 min
Hold	1	4°C	∞

3. Centrifuge the plate at 1,000 x *g* for 10 seconds, and then place it in a 96-well ice-cooled rack.

Preparing and pipetting the RT enzyme master mix to the plate

1. Place a 0.2 mL 12-tube strip on ice.
2. Thaw buffers and primers at room temperature and keep enzymes on ice.
3. In the pre-amplification workspace, in a new 1.5 mL LoBind tube, prepare the RT enzyme master mix by adding the components in this order:

Component	Volume per well (µL)	Volume for 96-well plate with overage (µL)
Precise RT Buffer (PN 650000039)	2.0	240
RNase Inhibitor (PN 650000040)	0.1	12.0
Precise Reverse Transcriptase (PN 650000041)	0.2	24.0
Total	**2.3**	**276.0**

4. Briefly vortex and centrifuge the mix.
5. Pipet 21 µL of the RT enzyme master mix into each well of the strip tubes that are on ice.
6. Slowly peel the seal from the Precise WTA Single Cell Encoding Plate to avoid contamination between wells.
7. Using new tips after each pipetting, use a 12-channel pipette to dispense 2.3 µL of the RT enzyme master mix into each of the 96 wells. The final RT reaction volume in each well is 10.0 µL.
8. Seal the plate with clear adhesive film, vortex for 10 seconds to mix the samples, and then centrifuge the plate at 1,000 x g for 10 seconds.

Performing reverse transcription

1. Run thermal cycler **program 2** in the pre-amplification thermal cycler:

Step	Cycles	Temperature	Time
Reverse transcription	1	42°C	30 min
Heat inactivation	1	80°C	5 min
Hold	1	4°C	∞

2. Remove the samples ≤60 minutes after thermal cycling.
3. Centrifuge the plate at 1,000 x g for 10 seconds, and then place it on ice.
4. To continue preparation of the Precise WTA library from RNA, proceed to Pooling the samples (page 32).