UST TELL-Seq™ Amplicon Phasing Library Prep User Guide

For PCR products (2-15 kb)

This User Guide is to be used with TELL-Seq™ Library Reagent Box 1 V1 which contains newly released Suspension Buffer EZ and TELL-Seq™ Library Reagent Box 2 V1 which includes TELL Bead Plex option.

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August 2022

This document is proprietary to Universal Sequencing Technology Corporation. It is intended solely for use with the products described herein and for no other purposes.

The instructions in this document must be followed precisely by adequately trained personnel to ensure the proper and safe use of the TELL-Seq™ kit.

UNIVERSAL SEQUENCING TECHNOLOGY CORPORATION DOES NOT ASSUME ANY LIABILITY OCCURRING AFTER INCORRECT USE OF THE TELL-SEQ™ KIT.

Revision History

Document Number	Date	Description of Change
Doc #100042 v1.0	July 2022	Initial Release
Doc #100042 v2.0	August 2022	Updated protocol to use with TELL-Seq™ Library Reagent V1 kits which contains Suspension Buffer EZ and TELL Bead Plex option.

Table of Contents

1. Introduction

This protocol explains the preparation of indexed paired-end TELL-Seq™ libraries using a TELL-Seq™ Library Prep Kit and one or more PCR-amplified regions (targeted amplicons) as input DNA material. TELL-Seq™ libraries are compatible with Illumina® sequencers.

The TELL-Seq™ Library Prep kit is based on a proven Transposase Enzyme-Linked Long-read Sequencing (TELL-Seq™) technology† that enables the generation of barcode-linked-reads in an Illumina® sequencing platform. Coupled with TELL-Seq™ analysis software, barcode-linked reads can be used to resolve haplotypes in PCR amplicons ranging in sizes from 2 kilobases (kb) to 15 kb and beyond.

Following this User Guide, a TELL-Seq™ Library Prep kit can:

- Generate up to 12 TELL-Seq™ libraries (Standard Size kit) or 72 TELL-Seq™ libraries (HT24 kit)
- Use as little as 0.02 ng (20 pg) or less of each amplicon as input material *
- Produce barcode-linked reads in an Illumina® sequencing system

** Note: TELL-Seq™ can process a single amplicon or multiple amplicons in the same reaction. For the panel (or pooled amplicons) option, it is a requirement that each amplicon must align to a different region in the genome (i.e., non-overlapping sequences). We estimate that a panel of up to 250 non-overlapping amplicons could be pooled in a single TELL-Seq™ reaction.*

Before You Start

Users should generate PCR amplicons and have clean PCR samples before starting this protocol. Below, we provide recommendations on generating and processing PCR amplicons suitable for phasing. We also provide recommendations on diluting DNA to picogram scale and other helpful information to consider before planning a TELL-Seq™ experiment.

Generating and handling PCR amplicons for targeted phasing

- Avoid PCR over-amplification. TELL-Seq™ requires picogram amounts of starting material. Thus, it is sufficient to amplify DNA to a level that is minimally detectable in an agarose gel stained with conventional methods (e.g., SYBR green or ethidium bromide staining).
- Allow long PCR extension steps. Be aware that incompletely extended molecules in one PCR cycle could be used as “primers” in the next PCR cycle. These partially extended molecules can form PCR chimeras when annealing to a DNA copy from which they did not originate (i.e., annealing to the maternal copy if they originated from the paternal copy, or annealing to the paternal copy if they originated from the maternal copy). Please, read the instructions provided by the manufacturer of the DNA polymerase used and double the recommended extension time in every cycle if possible.

† Patent pending.

- Use high-fidelity DNA polymerases. Also, use long-PCR DNA polymerase versions for >5kb amplicons (e.g., Supreme NZYLong DNA polymerase or an equivalent option).
- Amplicon purity and quality: It is recommended to work with a clean PCR amplicon that appears as a single band in an agarose gel, unless the extra-band sequences (if they exist) align to a non-targeted region in the genome. Also, use AMpure™ XL beads (Beckman Coulter©), ExoSAP-It™ (ThermoFisher©), or equivalent products to remove primer dimers.
- Quantification: Use a fluorometric-based method to quantify DNA amplicons, such as the Qubit™ dsDNA HS (high-sensitivity) kit and at least 2 µL of each DNA sample. Avoid methods that measure total nucleic acid, such as UV absorbance or NanoDrop™.
- Amplicon length: this kit has been developed to process amplicons in the range of 2 to 15 kb, but larger than 15kb fragments should perform similarly if PCR-chimera formation is minimized during PCR.

Diluting starting material at picogram scale

- Picogram amounts of DNA are used as starting material in TELL-Seq™ reactions, which often requires extensive dilution. Use fresh dilutions each time, including also fresh dilutions of lambda phage or *Escherichia coli* genomic DNA is this material is required (see below if adding non-human DNA is a requisite).
- When making dilutions, pipette gently to avoid DNA shearing, especially when handling large (>5kb) amplicons (e.g., avoid sample vortexing or excessive pipetting). Mix by inverting the tube when possible.
- Use low retention pipette tips and low-binding tubes when possible.
- Store stock amplicons and lambda phage or *Escherichia coli* genomic DNA preferably at high concentrations (not as a dilution) at 4°C or -20°C in a Tris buffer (pH 7.5 - 8.0).

Single-amplicon versus pooled amplicons TELL-Seq™ reactions

- We encourage pooling amplicons in a single TELL-Seq™ reaction over single-amplicon reactions. When pooling, however, each individual amplicon **must** align to a **different** region along the reference (i.e., they must represent **non-overlapping** genomic regions in the reference). Overlapping amplicons can only be pooled during sequencing—and if they do not share library index.

Adding lambda phage or Escherichia coli genomic DNA to a TELL-Seq™ reaction

- Adding lambda phage or *E. coli* genomic DNA is necessary when processing four or fewer non-overlapping amplicons in the same TELL-Seq™ reaction so that the total input DNA amount is 100pg (see table on Page 15 for additional details). Lambda phage or *E. coli* genomic DNA is not

part of the TELL-Seq™ Library Prep kit (see on Page 13, Equipment and Consumables Not Provided).

TELL-Seq™ Library Prep Kit Update Notification

We have made further enhancements to TELL-Seq™ library prep kits. TELL-Seq™ Library Reagent Box 1 V1 replaces previous Suspension Buffer with newly released Suspension Buffer EZ and TELL-Seq™ Library Reagent Box 2 V1 adds an optional bead type, TELL Bead Plex.

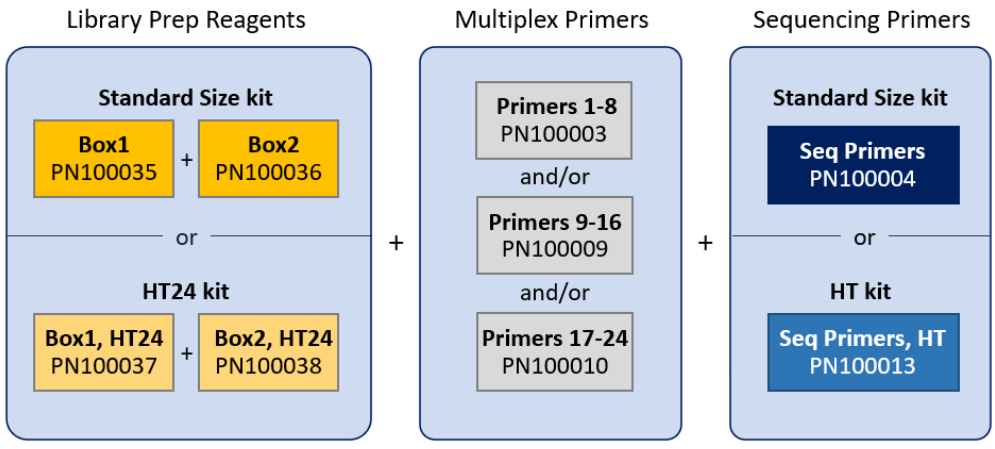
Suspension Buffer EZ has reduced viscosity and is easy to pipette accurately. It further improves library yield. Suspension Buffer EZ requires larger input volume than previous Suspension Buffer in a reaction.

TELL-Seq™ Library Reagent Box 2 V1 contains either standard TELL Bead or new TELL Bead Plex. TELL Bead Plex beads use modified linked read barcode (index 1) sequences and provide more balanced nucleotide signal at index 1 position 3, 6, 7, 12,13 and 16. While TELL-Seq™ libraries generated with standard TELL Bead produce excellent Index 1 sequencing quality on all Illumina systems, TELL-Seq™ libraries generated with TELL Bead Plex show excellent Index 1 sequencing quality on a variety of sequencing platforms including Illumina and non-Illumina systems.

TELL-Read pipeline v1.1 or above is required to analyze sequencing data generated from TELL-Seq™ libraries prepared with TELL Bead Plex and works for sequencing data generated from standard TELL Bead as well.

2. Kit contents

General overview: kit sizes, box combinations, and compatibilities



Comparing Library Prep Reagent kit sizes (Standard Size versus HT24)

The Standard Size kit can generate up to 12 TELL-Seq™ reactions; the HT24 Size kit can generate up to 72 TELL-Seq™ reactions.

- Thus, **six** Standard Size TELL-Seq™ Library Prep Kits (including each Box 1 and Box 2) are equivalent to **one** HT24 Size TELL-Seq™ Library Prep Kit (including Box 1 and Box 2)

Matching Library Prep Reagent and Multiplex Primer kits

Library Prep Reagent and Multiplex Primers kits can process a different number of TELL-Seq™ libraries. Here, we provide information on pairing these kits.

- **Four** Standard Size TELL-Seq™ Library Prep Kits (Box 1 and Box 2) can pair with **one** of any TELL-Seq™ Library Multiplex Primer Kits (either Primer Set 1-8, 9-16, or 17-24)
- **Two** HT24 Size TELL-Seq™ Library Prep Kits (Box 1 and Box 2) can pair with **three** of any TELL-Seq™ Library Multiplex Primer Kits (either Primers 1-8, 9-16, and/or 17-24)

Sequencing Primer kits

- Custom sequencing primers are required for sequencing TELL-Seq™ libraries. The minimum number of sequencing runs that can be performed using the amount of sequencing primer provided in the Sequencing Primer Kits varies based on the sequencing system as follows

Sequencing System	Number of runs (Standard Size kit)	Number of runs (HT24 kit)	Is custom Index 2 Primer required?
NovaSeq	4	24	v1 reagent: No; v1.5 reagent: Yes
HiSeq 3000/4000	2	12	Yes
HiSeq 2000/2500	5	30	No
NextSeq	8	48	Yes
MiSeq	16	96	No
MiniSeq	8	48	Yes

TELL-Seq™ Library Prep Kit, Standard Size **(2 Boxes)**

Box 1 of 2: TELL-Seq™ Library Reagent Box 1 **(PN 100035)**

NOTE: Do not freeze and thaw Box 1 reagents for more than 6 times.

Component Name	Cap Color	Volume (µL) [a]	Storage Temperature
5× Reaction Buffer	CAP Blue	120	-25°C to -15°C
Barcoding Enzyme	CAP Black	24	-25°C to -15°C
Cofactor II	CAP Amber	120	-25°C to -15°C
Exonuclease	CAP Yellow	12	-25°C to -15°C
Stabilizer	CAP Violet	12	-25°C to -15°C
Suspension Buffer EZ	CAP Natural	180	-25°C to -15°C
Tagging Enzyme	CAP Red	24	-25°C to -15°C
2× PCR Master Mix	CAP Pink	150	-25°C to -15°C
Enhancer	CAP Green	18	-25°C to -15°C
10× Primer I [b]	CAP White	30	-25°C to -15°C

[a] Guaranteed volumes (excess volume is expected)

[b] For use with 10× Primer II in any TELL-Seq™ Library Multiplex Primer Kit together for library amplification.

Box 2 of 2: TELL-Seq™ Library Reagent Box 2 **(PN 100036)**

Component Name	Cap Color	Volume (µL) [a]	Storage Temperature
TELL-Bead or TELL Bead Plex [b]	CAP Orange	76	2°C to 8°C
Wash Solution	CAP White	4500	2°C to 8°C
Stop Solution [c]	CAP Natural	960	2°C to 25°C

[a] Guaranteed volumes (excess volume is expected)

[b] TELL Bead works well on Illumina Sequencing Systems; TELL Bead Plex works well on both Illumina and non-Illumina Sequencing System.

[c] Before use, if the Stop Solution is not clear, warm the tube up at 37°C. Vortex to dissolve any precipitate. After the first use, store resuspended Stop Solution at room temperature for future use.

CAUTION

TELL-Read pipeline v1.1 or above is required to analyze sequencing data generated from TELL-Seq™ libraries prepared with TELL Bead Plex and works for data generated from standard TELL Bead as well.

TELL-Seq™ Library Prep Kit, HT24 **(2 Boxes)**

Box 1 of 2: TELL-Seq™ Library Reagent Box 1, HT24 **(PN 100037)**

NOTE: Do not freeze and thaw Box 1 reagents for more than 6 times.

Component Name	Cap Color	Volume (µL) [a]	Storage Temperature
5× Reaction Buffer	CAP Blue	720	-25°C to -15°C
Barcoding Enzyme	CAP Black	144	-25°C to -15°C
Cofactor II	CAP Amber	720	-25°C to -15°C
Exonuclease	CAP Yellow	72	-25°C to -15°C
Stabilizer	CAP Violet	72	-25°C to -15°C
Suspension Buffer EZ	CAP Natural	1080	-25°C to -15°C
Tagging Enzyme	CAP Red	144	-25°C to -15°C
2× PCR Master Mix	CAP Pink	900	-25°C to -15°C
Enhancer	CAP Green	108	-25°C to -15°C
10× Primer I [b]	CAP White	180	-25°C to -15°C

[a] Guaranteed volumes (excess volume is expected)

[b] For use with 10× Primer II in the TELL-Seq™ Library Multiplex Primer Kit together for library amplification.

Box 2 of 2: TELL-Seq™ Library Reagent Box 2, HT24 **(PN 100038)**

Component Name	Cap Color	Volume [a]	Storage Temperature
TELL-Bead or TELL Bead Plex [b]	CAP Orange	456 µL	2°C to 8°C
Wash Solution	CAP Blue	28.5 mL	2°C to 8°C
Stop Solution [c]	CAP White	5.76 mL	2°C to 25°C

[a] Guaranteed volumes (excess volume is expected)

[b] TELL Bead works well on Illumina Sequencing Systems; TELL Bead Plex works well on both Illumina and non-Illumina Sequencing System.

[c] Before use, if the Stop Solution is not clear, warm the tube up at 37°C. Vortex to dissolve any precipitate. After the first use, store resuspended Stop Solution at room temperature for future use.

CAUTION

TELL-Read pipeline v1.1 or above is required to analyze sequencing data generated from TELL-Seq™ libraries prepared with TELL Bead Plex and works for data generated from standard TELL Bead as well.

TELL-Seq™ Library Multiplex Primer (1-8) Kit **(PN 100003)**

Component Name	Cap Color	Volume (µL) [a]	Storage Temperature
10× Primer II, T501	CAP Blue	15	-25°C to -15°C
10× Primer II, T502	CAP Black	15	-25°C to -15°C
10× Primer II, T503	CAP Green	15	-25°C to -15°C
10× Primer II, T504	CAP Yellow	15	-25°C to -15°C
10× Primer II, T505	CAP Violet	15	-25°C to -15°C
10× Primer II, T506	CAP Natural	15	-25°C to -15°C
10× Primer II, T507	CAP Red	15	-25°C to -15°C
10× Primer II, T508	CAP Orange	15	-25°C to -15°C

[a] Guaranteed volumes (excess volume is expected)

TELL-Seq™ Library Multiplex Primer (9-16) Kit **(PN 100009)**

Component Name	Cap Color	Volume (µL) [a]	Storage Temperature
10× Primer II, T509	CAP Blue	15	-25°C to -15°C
10× Primer II, T510	CAP Amber	15	-25°C to -15°C
10× Primer II, T511	CAP Green	15	-25°C to -15°C
10× Primer II, T512	CAP Yellow	15	-25°C to -15°C
10× Primer II, T513	CAP Violet	15	-25°C to -15°C
10× Primer II, T514	CAP Orange	15	-25°C to -15°C
10× Primer II, T515	CAP Red	15	-25°C to -15°C
10× Primer II, T516	CAP Natural	15	-25°C to -15°C

[a] Guaranteed volumes (excess volume is expected)

TELL-Seq™ Library Multiplex Primer (17-24) Kit **(PN 100010)**

Component Name	Cap Color	Volume (µL) [a]	Storage Temperature
10× Primer II, T517	CAP Amber	15	-25°C to -15°C
10× Primer II, T518	CAP Blue	15	-25°C to -15°C
10× Primer II, T519	CAP Yellow	15	-25°C to -15°C
10× Primer II, T520	CAP Green	15	-25°C to -15°C
10× Primer II, T521	CAP Black	15	-25°C to -15°C
10× Primer II, T522	CAP Violet	15	-25°C to -15°C
10× Primer II, T523	CAP Orange	15	-25°C to -15°C
10× Primer II, T524	CAP Red	15	-25°C to -15°C

[a] Guaranteed volumes (excess volume is expected)

TELL-Seq™ Illumina® Sequencing Primer Kit **(PN 100004)**

Component Name	Cap Color	Concentration	Volume (µL) [a]	Storage Temperature
Read 1 Primer	CAP Black	100µM	50	-25°C to -15°C
Read 2 Primer	CAP White	100µM	50	-25°C to -15°C
Index 1 Primer	CAP Red	100µM	50	-25°C to -15°C
Index 2 Primer	CAP Yellow	100µM	50	-25°C to -15°C

[a] Guaranteed volumes (excess volume is expected)

TELL-Seq™ Illumina® Sequencing Primer Kit, HT **(PN 100013)**

Component Name	Cap Color	Concentration	Volume (µL) [a]	Storage Temperature
Read 1 Primer	CAP Black	100µM	300	-25°C to -15°C
Read 2 Primer	CAP White	100µM	300	-25°C to -15°C
Index 1 Primer	CAP Red	100µM	300	-25°C to -15°C
Index 2 Primer	CAP Yellow	100µM	300	-25°C to -15°C

[a] Guaranteed volumes (excess volume is expected)

3. Consumables and Equipment (not provided)

Consumables

Consumable	Supplier
0.2 mL PCR tube or strip tube	General lab supplier
20 µL pipette tip (standard and wide orifice)	General lab supplier
200 µL pipette tip (standard and wide orifice)	General lab supplier
Lambda phage DNA [a]	New England Biolabs, #N3011S
Ethanol 200 proof (absolute) for molecular biology (500 mL)	Sigma-Aldrich, # E7023
Nuclease-free water	General lab supplier
AMPure XP	Beckman Coulter, # A63880
Agilent Bioanalyzer High Sensitivity DNA Analysis Kit [b]	Agilent, # 5067-4626
TapeStation High Sensitivity D5000 ScreenTape Assay [b]	Agilent, # 5067-1506
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific, # Q32851 or Q32854
	Thermo Fisher Scientific, # Q32856
TE buffer, pH 8.0	General lab supplier

[a] Alternatively, *Escherichia coli* genomic DNA can also be used.

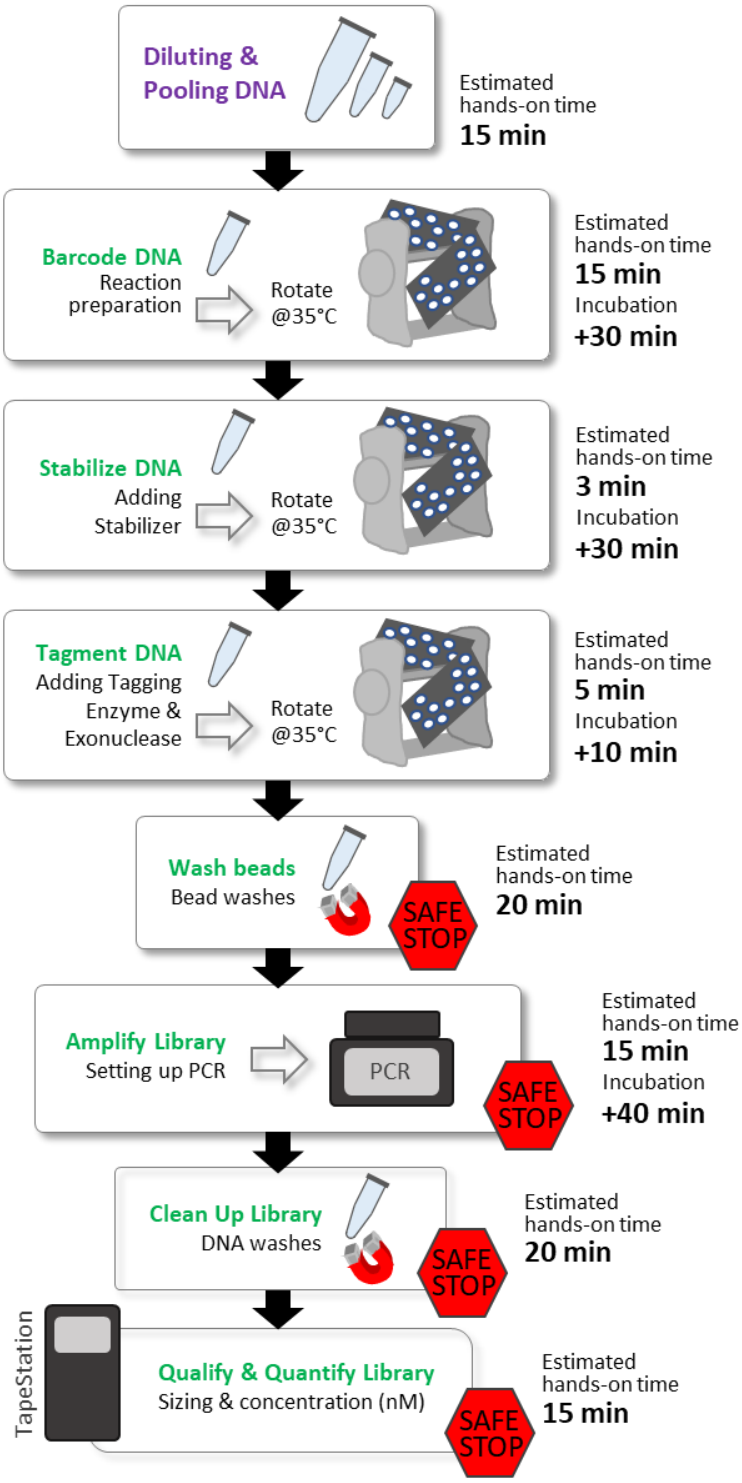
[b] One or the other instrument (two options), depending on the DNA quantification system available in the user's lab or facility.

Equipment

Equipment	Supplier
Thermocycler	Applied Biosystems
Magnetic stand for 0.2 mL PCR tubes	General lab supplier
Tube Rotator	General lab supplier
Incubator (for 35°C)	General lab supplier
Vortexer	General lab supplier
Microcentrifuge	General lab supplier
Agilent Bioanalyzer [a]	Agilent
Agilent TapeStation [a]	Agilent
Qubit® Fluorometer 3.0 or higher	Thermo Fisher Scientific, # Q33216 or Q33238
Ice Bucket	General lab supplier

[a] One or the other instrument (two options), depending on the DNA quantification system available in the user's lab or facility.

4. TELL-Seq™ Library Prep Workflow



5. Protocol

This protocol is designed for targeted phasing applications using PCR amplicons as input material. TELL-Seq™ Library Prep kits are designed to generate up to 12 TELL-Seq™ amplicon libraries using Standard Size kits and up to 72 TELL-Seq™ libraries using HT24 kits. A single amplicon or multiple amplicons can be processed in a single TELL-Seq™ reaction to generate one TELL-Seq™ library (referred to as single-amplicon and pooled amplicons or panel options, respectively).

IMPORTANT NOTE: The panel option can only work if individual amplicons align to different regions along the genome (i.e., non-overlapping sequences).

The following protocol describes library preparation procedures (see workflow and safe stopping points on Page 13).

Barcode diluted DNA

I. Consumables

- Input DNA (User)

Number of amplicons (n)	Amplicon amount (x, pg) [a]	Lambda phage DNA amount (pg) [b]	Reaction Vol (µL)	Preps/ Standard Size Kit	Preps/ HT24 Kit
n=1 (single-amplicon option)	x = 20	80	50	12	72
n=2-4 (panel option) [c]	x = 20 * n (20 each)	100 – x	50	12	72
n=5 or more (panel option) [c]	x= 100/n (each)	--	50	12	72

NOTES:

a) DNA should be stored and diluted in a Tris buffer with pH ranging from 7.5 to 8.0 or a low TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Preferably, dilutions should be made fresh.

b) Lambda phage genomic DNA (or *E. coli* genomic DNA) is added to TELL-Seq reactions so that the total input DNA amount is 100 pg. In single-amplicon reactions, 20 pg of the amplicon is processed in combination with 80 pg of lambda phage or *E. coli* genomic DNA. With more than one amplicon in a single TELL-Seq reaction (panel option), volumes are adjusted as indicated in the above table.

c) For panels (non-overlapping sequences), equal amounts of each amplicon should be used regardless of fragment size. There will be a larger number of short amplicon molecules compared to long amplicon molecules for the same amount of DNA, but the linked-read efficiencies will be higher for long amplicons compared to short amplicons; together, balancing out. Related to amplicon length, this kit has been developed to process amplicons in the range of 2 to 15 kb, but larger than 15kb fragments should perform similarly if PCR-chimera formation is minimized.

- 5× Reaction Buffer (Kit Box 1, CAP Blue)
- Cofactor II (Kit Box 1, CAP Amber)
- Barcoding Enzyme (Kit Box 1, CAP Black)
- TELL Bead or TELL Bead Plex (Kit Box 2, CAP Orange)
- Suspension Buffer EZ (Kit Box 1, CAP Natural)
- Nuclease-free water (User)
- 0.2 mL PCR tube or strip tube (User)
- 20 µL and 200 µL wide orifice pipette tips (User)

II. Preparation

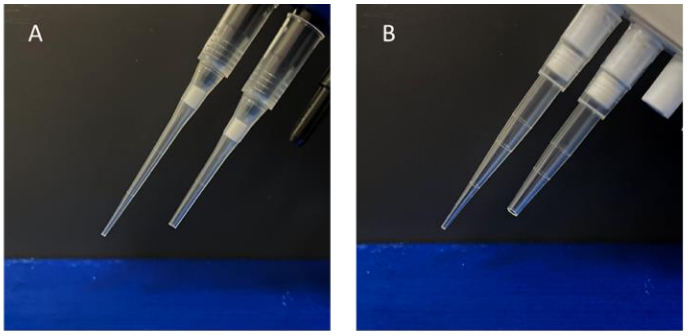
1. Prepare the following consumables:

Item	Storage	Instruction
5× Reaction Buffer CAP	-25°C to -15°C	Thaw at room temperature. Vortex to mix, then centrifuge briefly. Keep on ice.
Cofactor II CAP	-25°C to -15°C	Vortex to mix, then centrifuge briefly. Keep at **room temperature** but **avoid light. Close the tube cap tightly after each use.**
Barcoding Enzyme CAP	-25°C to -15°C	Centrifuge briefly. Keep on ice.
TELL Bead or TELL Bead Plex CAP	2°C to 8°C	Centrifuge briefly. Keep on ice. **Close the tube cap tightly after each use** to avoid any evaporation.
Suspension Buffer EZ CAP	-25°C to -15°C	Thaw at room temperature. Vortex to mix, then centrifuge briefly. Keep at **room temperature**.
Nuclease-free water	Room Temperature	Keep at room temperature.

2. Set up a tube rotator inside an incubator (e.g., image below). Set the temperature in the incubator to be at 35°C for at least 30 minutes prior to starting the procedure.

CAUTION

Use wide-orifice pipette tips to mix amplicons to avoid shearing of DNA. If wide-orifice pipette tips are not available, then cut 2mm-3mm off of the end of the tip with a clean razor blade or scissors before use (see image below). For sample transfer between tubes, use standard uncut tips for volume accuracy.



A. 20uL pipette tips (uncut and cut) B. 200uL pipette tips (uncut and cut)

3. Preparing the input DNA material*:

- TELL-Seq™ reactions use picogram-scale amounts of DNA, which requires preparing highly diluted input samples for both input and competitor DNA (such as lambda phage or *Escherichia coli* genomic DNA). Making ultra-high dilutions requires best laboratory practices, including minimizing the number of diluting steps and using large DNA volumes when possible. For instance, two diluting steps would be better than three diluting steps and diluting 3-5 μL of DNA would be better than diluting 1 μL, even if it requires mixing the solutions in large 15 mL tubes.
- We recommend using low binding tubes, especially with the most diluted solutions.
- Making ultra-high dilutions of amplicons, especially for kilobase-long fragments, requires thoroughly but gently mixing (for example, by inverting tube >20 times or by pipetting using wide-orifice or cut tips to avoid shearing the DNA).
- Total input DNA should be 100 pg. For a single amplicon or pools of 2 to 4 amplicons, 20 pg of each amplicon must be added, and the difference should be made up to 100 pg using lambda phage or *E. coli* genomic DNA (also referred to as "competitor DNA"). When pooling 5 or more amplicons, no lambda phage or *E. coli* genomic DNA should be added; the amount of each amplicon will be calculated by dividing the total number of amplicons by 100 (e.g., 16.66 μL of each amplicon when pooling 6 amplicons).
- It is highly recommended to make fresh dilutions of the amplicon and lambda phage or *E. coli* genomic DNA before TELL-Seq™ library prep as the DNA does not store well at ultra-low concentrations.

* User should have generated PCR amplicons before starting this protocol (refer to *Before You Start* section on Page 5 for suggestions).

III. Procedure

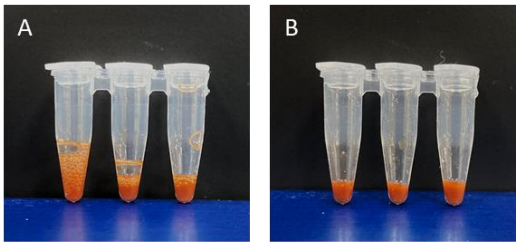
1. Vortex TELL Bead or TELL Bead Plex vigorously for at least 30 seconds. Pulse spin for no more than 1 second to collect any beads present on the lid or walls of the tube. Right before use, using a P200, pipet the TELL Bead or TELL Bead Plex up and down 5 times to ensure all the beads are resuspended thoroughly.
2. In a **0.2 mL PCR tube**, assemble each reaction in the following order. (Mix enzymes before use by flicking the tube several times and centrifuge briefly afterward. Read instructions in the table in Section II.1 on Page 15).

Reagent	Volume per reaction (50 µL)
5× Reaction Buffer CAP	10
Nuclease-free water	7 – x (x is the total DNA volume) (See the table on Page 14)
Cofactor II CAP	10
TELL Bead or TELL Bead Plex CAP (0.5M barcodes/µL)	6

3. Mix well by pipetting or vortexing vigorously and centrifuge for 1 second to bring the solution down to the bottom.
4. Flick mix the vial containing the Barcoding Enzyme 3-4 times, then pulse centrifuge. Add Barcoding Enzyme:

Reagent	Volume per reaction (µL)
Barcoding Enzyme CAP	2

4. Mix well by pipetting up and down 5 times. Avoid introducing air bubbles by keeping the pipette tip at the bottom of the solution during mixing.



A. Bubble issue B. Properly mixed

5. Add the following reagents (first, read instructions in the table in Section II.1 on Page 15).

IMPORTANT NOTE: Suspension Buffer is highly viscous and must be thoroughly mixed prior to use. Vortex the Suspension Buffer thoroughly, and then pulse centrifuge. Use caution to ensure that correct volume is delivered while pipetting.

Reagent	Volume per reaction (μL)
Sample DNA (amplicon/s + lambda phage or *E. coli* genomic DNA if required)	x μL (≤ 5 μL)
Suspension Buffer CAP	15

6. Use a wide orifice pipette tip, set pipette volume to 35 μL, and gently mix the solution by **slowly** pipetting up and down 6-8 times.

   IMPORTANT NOTE: Avoid introducing air bubbles by keeping the pipette tip at the bottom of the solution when pipetting.

7. Place the tube onto a tube rotator in a 35°C incubator and rotate slowly (10-15 rpm) for 30 minutes.

Stabilize DNA

I. Consumables

- Stabilizer (Kit Box 1, CAP Violet)

II. Preparation

- Prepare the following consumables:

Item	Storage	Instruction
Stabilizer CAP	-25°C to -15°C	Centrifuge briefly. Keep on ice.

- Use the same tube rotator in the 35°C incubator.

III. Procedure

1. Take the sample tube out of the 35°C incubator.
2. Add the Stabilizer into the tube.

Reagent	Volume per reaction (μL)
Stabilizer CAP	1

3. Use a wide orifice pipette tip, set pipette volume to 35 μL; gently mix the solution by **slowly** pipetting up and down 6-8 times.

   IMPORTANT NOTE: Avoid introducing air bubbles by keeping the pipette tip at the bottom of the solution when pipetting.

4. Place the sample tube back on the tube rotator in the 35°C incubator and rotate it slowly (10-15 rpm) for 30 minutes.

Tagment DNA

IV. Consumables

- Tagging Enzyme (Kit Box 1, CAP Red)
- Exonuclease (Kit Box 1, CAP Yellow)

V. Preparation

1. Prepare the following consumables:

Item	Storage	Instruction
Tagging Enzyme CAP	-25°C to -15°C	Centrifuge briefly. Keep on ice.
Exonuclease CAP	-25°C to -15°C	Centrifuge briefly. Keep on ice.

2. Use the same tube rotator in the 35°C incubator.

VI. Procedure

1. Take the sample tube out of the 35°C incubator.
2. Add Tagging Enzyme and Exonuclease into the tube.

Reagent	Volume per reaction (µL)
Tagging Enzyme CAP	1
Exonuclease CAP	1

3. Use a wide orifice pipette tip, set the volume to 35 µL; gently mix the solution by **slowly** pipetting up and down 8 times.

   IMPORTANT NOTE: For this step, the mixing needs to be very thorough. The pipetting does NOT need to be as gentle as in the Barcoding and Stabilizing DNA steps but, still, avoid introducing air bubbles by keeping the pipette tip at the bottom of the solution when pipetting.

4. Place the tube back on the tube rotator in the 35°C incubator and rotate it slowly (10-15 rpm) for 10 minutes.
5. Proceed to the next step immediately after the incubation.

Wash Beads

I. Consumables

- Stop Solution (Kit Box 2, CAP Natural in the standard size kit, CAP White in the HT24 kit, or stored at room temperature after the first use)
- Wash Solution (Kit Box 2, CAP White in the standard size kit, CAP Blue in the HT24 kit)
- 0.2 mL PCR tube or strip tube (User)

II. Preparation

1. Prepare the following consumables:

Item	Storage	Instruction
Stop Solution	2°C to 25°C	Check for any precipitates. If present, incubate the buffer at 37°C for 10 minutes and vortex until they dissolve. Keep at room temperature for future use.
Wash Solution	2°C to 8°C	Bring to room temperature.

2. Set up a thermocycler with the following program:
    - Preheat lid option to 100°C
    - 63°C forever

III. Procedure

1. Place the sample tube on a magnetic stand for 1 minute or until the solution is clear.
2. While the tube is on the magnetic stand, aspirate and discard the supernatant without disturbing beads. Use a P20 pipette to remove any remaining supernatant.
3. Remove the tube from the magnetic stand. Add 110 µL Wash Solution to the PCR tube. Pipet to resuspend the beads. If necessary, briefly centrifuge (~1 second spin) to bring the solution down.
4. Place the sample tube back on the magnetic stand for 1 minute or until the solution is clear.
5. While the tube is on the magnetic stand, aspirate and discard the supernatant without disturbing beads. Use a P20 pipette to remove any remaining supernatant.
6. Remove the tube from the magnetic stand. Add 80 µL of Stop Solution to the tube.
7. Pipet up and own five times to resuspend the beads. If necessary, bring the solution down with a quick ~1 second spin in the centrifuge.
8. Incubate the tube at room temperature for 5 minutes.
9. Place the sample tube back on the magnetic stand for 1 minute or until the solution is clear.
10. While the tube is on the magnetic stand, aspirate and discard the supernatant without disturbing beads. Use a P20 pipette to remove any remaining supernatant.
11. Remove the tube from the magnetic stand. Add 110 µL Wash Solution to the PCR tube. Pipet to resuspend the beads.
12. **Transfer the entire bead solution into a new 0.2 mL PCR tube.**
13. Incubate the tube at 63° C on the PCR thermocycler for 3 minutes.

14. Place the sample tube on the magnetic stand at room temperature for 1 minute or until the solution is clear.
15. While the tube is on the magnetic stand, aspirate and discard the supernatant without disturbing beads.
16. Remove the tube from the magnetic stand. Add 110 µL Wash Solution to the PCR tube. Pipet to resuspend the beads. Centrifuge for 1 second to bring all solution down to the bottom of the PCR tube when necessary.
17. Incubate the tube at 63° C on the PCR thermocycler for 3 minutes.
18. Put the PCR tube on the magnetic stand at room temperature for 1 minute or until the solution is clear.
19. While the tube is on the magnetic stand, aspirate and discard the supernatant without disturbing beads. Use a P20 pipette to remove any remaining supernatant.
20. Remove the tube from the magnetic stand. Resuspend the beads in 40 µL of Wash Solution.

> NOTE:
>
> This is a **SAFE STOPPING POINT**. The washed beads can be stored at 2°C to 8°C for two weeks.

Amplify Library

I. Consumables

- 2× PCR Master Mix (Kit Box 1, CAP Pink)
- 10× Primer I (Kit Box 1, CAP White)
- 10× Primer II, T5## (Multiplex Primer Kit)
- Nuclease-free water (User)
- 0.2 mL PCR tube or strip tube (User)

II. Preparation

1. Prepare the following consumables:

Item	Storage	Instruction
2× PCR Master Mix CAP	-25°C to -15°C	Thaw at room temperature. Mix gently, then centrifuge briefly. Keep on ice.
10× Primer I CAP	-25°C to -15°C	Thaw at room temperature. Vortex to mix, then centrifuge briefly. Keep on ice.
10× Primer II, T5##	-25°C to -15°C	Thaw at room temperature. Vortex to mix, then centrifuge briefly. Keep on ice.
Enhancer CAP	-25°C to -15°C	Thaw at room temperature. Vortex to mix, then centrifuge briefly. Keep at room temperature.
Nuclease-free water	Room Temperature	Keep at room temperature.

2. Set up Library Amplification Program (**LAP**) on a thermocycler as follows:
    - 2 minutes at 63°C (1 cycle)
    - 2 minutes at 72°C (1 cycle)
    - 30 seconds at 98°C (1 cycle)
    - [15 seconds at 98°C, 20 seconds at 63°C, 30 seconds at 72°C] x 21 cycles*
    - 3 minutes at 72°C (1 cycle)
    - forever at 4°C

    ***** The final library concentration after cleanup (Page 24) should be 2-4nM. If getting lower or higher concentrations, the user could repeat the PCR using beads from Step 20 on Page 21 and a higher or lower number of PCR cycles. However, although we generally provide an excess of reagents that could be used for this purpose, we can only guarantee PCR reagents for the number of TELL-Seq™ reactions indicated in the kit.

> NOTE:
>
> Compromising between volume of processed TELL Beads and sequencing depth - Deep sequencing of low number of TELL Beads will lead to a higher linked read density and better performance. However, processing too few TELL Beads for library amplification can lead to low library complexity and high sequencing read duplication.

III. Procedure

1. Vortex beads vigorously for 10 seconds or longer if necessary (i.e., until beads are fully resuspended). Pulse spin to collect any beads present on the lid or walls of the tube.
2. Using a 20 μL tip, pipet the beads up and down 5 times to ensure all the beads are resuspended properly. Immediately transfer 1 μL of the bead solution to a new PCR tube. Keep the rest (19 μ) at 4°C or -20°C (in case the user wants to repeat the PCR in the future).

    IMPORTANT NOTE: The TELL-Seq™ Library Prep kit provides enough reagents for the specified number of TELL-Seq™ reactions (12 in the Standard size kit and 72 for the HT24 kit), although generally, it comes with an excess of reagents, but there is no guarantee of a particular number of additional PCR reactions that can be performed.

3. Add the following reagents to the PCR tube containing 1 μL of bead solution.

Reagent	Volume per reaction (μL)
Nuclease-free water	5
2× PCR Master Mix CAP	12.5
10× Primer I CAP	2.5
10× Primer II, T5##	2.5
Enhancer CAP	1.5

4. Mix well by pipetting. Bring solution down with a quick ~1 second spin in the centrifuge.
5. Place the tube on the thermal cycler and run the **LAP** program (see above) with 21 cycles.
6. After PCR amplification, save 2 μL PCR product for quality check on a Bioanalyzer or a TapeStation. See Qualify and Quantify Library section for instruction.

> NOTE:
>
> This is a **SAFE STOPPING POINT**. The PCR product can be stored at -25°C to -15°C for one month.

Clean Up Library

I. Consumables

- AMPure XP (User)
- Ethanol 200 proof (absolute) for molecular biology (User)
- Nuclease-free water (User)
- TE buffer, pH 8.0 (User)
- 0.2 mL PCR tube or strip tube (User)

II. Preparation

1. Prepare the following consumables:

Item	Storage	Instruction
Fresh 75% (v/v) ethanol	Room Temperature	Require 400 μL per sample. Prepare by mixing 1.5 mL Ethanol (200 proof) with 0.5 mL Nuclease-free water. Vortex to mix and keep at room temperature.
AMPure XP	2°C to 8°C	Please bring it to room temperature for at least 20 minutes and vortex vigorously to resuspend the beads before use.
Nuclease-free water	Room Temperature	Keep at room temperature.
TE buffer, pH 8.0	Room Temperature	Keep at room temperature.

III. Procedure

1. Bring solution down with a quick ~1 second spin in the centrifuge.
2. Place the sample tube on the magnetic stand for 1 minute or until the solution is clear.
3. While the tube is on the magnetic stand, transfer the supernatant to a new 0.2 mL PCR tube without disturbing beads.
4. Measure the volume of transferred supernatant (PCR product) with a pipette.
5. Add the following reagents into the PCR product to a total volume of 100 μL.

Reagent	Volume per reaction
PCR product	20 to 24 μL
Nuclease-free water	To final 100 μL total

6. Vortex vigorously to resuspend the AMPure XP solution and add 78 μL AMPure XP into the 100 μL PCR product.
7. Mix by pipetting up and down 10 times.
8. Incubate at room temperature for 5 minutes.
9. Place the tube on the magnetic stand for 1 minute or until the solution is clear.
10. Aspirate and discard the supernatant without disturbing AMPure beads.
11. While keeping the tube on the magnetic stand, add 200 μL of freshly prepared 75% ethanol into the tube, and wait for 30 seconds.
12. Aspirate and discard the supernatant without disturbing the beads.
13. Repeat steps 11-12 one more time, keeping the tube on the magnetic stand for the whole time. Use a P20 pipette to remove any remaining supernatant.
14. Leave the tube on the magnetic stand with the cap open and allow the tube to dry for 1-2 minutes to evaporate traces of ethanol. **DO NOT OVER-DRY THE BEADS.**
15. Take the tube off the magnetic stand and add 22 μL TE buffer to the beads.
16. Pipette or vortex to resuspend the beads. Let it sit for 5 minutes at room temperature.
17. Place the sample tube on the magnetic stand for 1 minute or until the solution is clear.
18. With the sample tube still on the magnetic stand, recover 20 μL of the supernatant to a new tube. Be careful not to disturb the beads.
19. The supernatant contains the TELL-Seq™ library.

> NOTE:
>
> This is a **SAFE STOPPING POINT**. The purified TELL-Seq library can be stored at -25°C to -15°C for six months.

Qualify and Quantify Library

I. Consumables

- Agilent High Sensitivity DNA Kit **or** TapeStation High Sensitivity D5000 ScreenTape Assay (User)
- Qubit dsDNA HS Assay Kit (User)
- TE buffer, pH 8.0 (User)

> NOTE:
>
> Standard qPCR library quantitation assay for Illumina system works for TELL-Seq library, but it is not required.

II. Preparation

1. Prepare the necessary consumables as required by Bioanalyzer or TapeStation and Qubit.

III. Procedure

1. Use 1 µL of the AMPure purified library for Agilent High Sensitivity DNA Kit or 2 µL of the library for TapeStation High Sensitivity D1000 or D5000 ScreenTape Assay.
2. Check the saved uncleaned PCR product from the Amplify Library section simultaneously. Uncleaned PCR products may have a higher level of primer-dimer and adapter dimer. It requires a two-fold dilution with nuclease-free water before loading onto a Bioanalyzer chip or TapeStation tape to avoid interfering with the lower marker signal.
3. To determine the library concentration, set the Region on the Bioanalyzer or TapeStation analysis software from 150 bp to 1000 bp. Record sample Concentration (nM) for this region (see Figure 1). At least 1 nM should be expected. To determine the library size, set the Region from 150 bp to 3000 bp – record sample Average Size (bp) as Library Size. A good-sized library should have most library fragments under 1000 bp.

CAUTION

The concentration reading from the Bioanalyzer (or TapeStation) should be used as a starting point to make necessary dilution or library pooling for sequencing. Verify the concentration of the final diluted sequencing library or library pool with a Qubit dsDNA HS Assay kit (see Step 6, below).

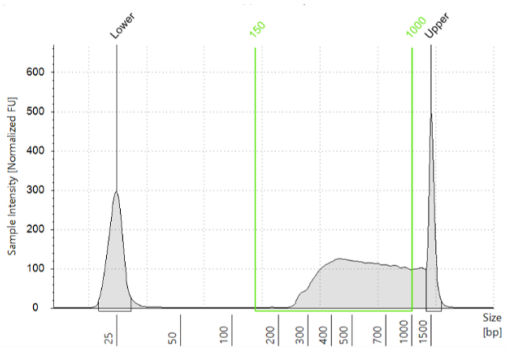


Figure 1. An example of a cleaned-up library profile from a TapeStation High Sensitivity D1000 ScreenTape assay.

4. The library can be sequenced immediately or stored at -25°C to -15°C.

NOTE:

Occasionally, there is a detectable residual level of adapter dimer in a cleaned-up library (see Figure 2). An additional round of AMPure XP cleanup as described in Clean Up Library section is recommended in this case.

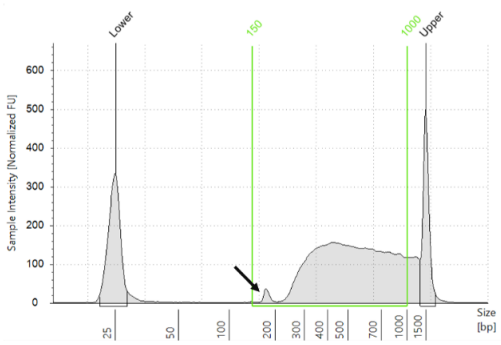


Figure 2. A library with detectable residual adapter dimer (arrow) after cleanup (TapeStation High Sensitivity D1000 ScreenTape assay).

5. When sequencing, dilute the library using TE buffer to the concentration recommended by each Illumina® sequencing system. Make a diluted library pool for sequencing if more than one library will be sequenced in the same run.
6. Use 4 µL of **diluted** sequencing library or library pool to check the concentration with the Qubit dsDNA HS Assay Kit. Use the Library Size value measured from the Bioanalyzer (or TapeStation) to convert mass concentration into molar concentration.

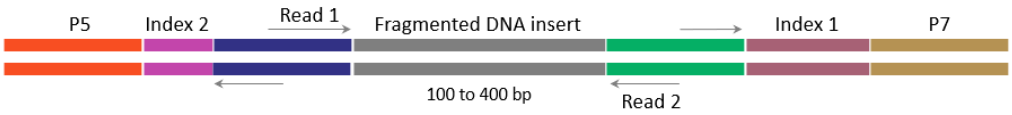
   A = Mass Concentration (ng/µL)
   S = Library Size (bp)

   Molar Concentration (nM) = (A*1,000,000)/(S*650)

7. Adjust the volume needed in the sequencing preparation if the library concentration measured by Qubit differs from the recommended concentration by more than 10%.

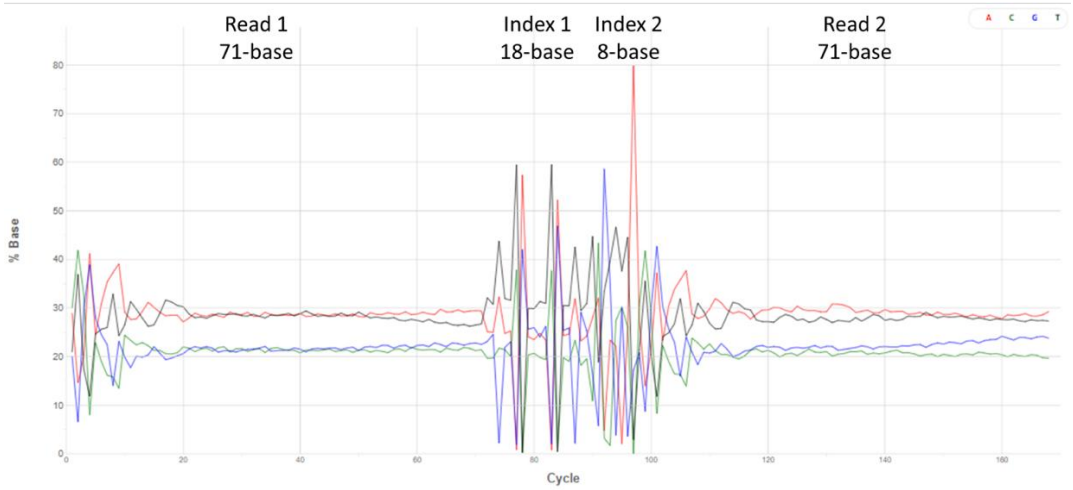
6. Appendix

TELL-Seq™ Library Structure and Sequencing Scheme



Index 1 contains 18-base TELL Bead sequences, which must be sequenced entirely. Index 2 contains 8-base (T-series) sample index primer sequences used in library amplification. Paired-end sequencing is preferred. The minimum read length requirement is 2x96; the maximum is 2x150.

Example of Illumina® Sequencing % Base by Cycle Chart



Illumina® Sequencing Guide

1. Dilute TELL-Seq™ library according to Illumina® sequencing platform-specific concentration and volume.
2. Libraries may be pooled together for sequencing when different multiplex primers are used in the library amplification step.
3. Custom sequencing primers are required to sequence TELL-Seq™ libraries and are provided in the TELL-Seq™ Illumina Sequencing Primer Kit.

TELL-Seq™ Illumina Sequencing Primer Kits

Component Name	Concentration	Storage Temperature
Read 1 Primer	100 μM	-25°C to -15°C
Read 2 Primer	100 μM	-25°C to -15°C
Index 1 Primer	100 μM	-25°C to -15°C
Index 2 Primer	100 μM	-25°C to -15°C

4. These custom sequencing primers can be loaded into the specified wells for custom primers. Alternatively, they can be loaded into corresponding standard Illumina® sequencing primer wells when an Illumina® PhiX control library is spiked into a sequencing run.
5. Custom Index 2 primer is only needed when multiple TELL-Seq™ libraries with different multiplex primers are pooled for sequencing and when a sequencer requires an i5 index sequencing primer. **For MiSeq, HiSeq 2000/2500, and NovaSeq v1 reagents, custom Index 2 Primer is not required.**
6. The minimum number of sequencing runs that can be performed using the amount of sequencing primer provided varies based on the sequencing system.

Sequencing System	Is custom Index 2 Primer required?
NovaSeq	v1 reagent: No; v1.5 reagent: Yes
HiSeq 3000/4000	Yes
HiSeq 2000/2500	No
NextSeq	Yes
MiSeq	No
MiniSeq	Yes

Illumina® Sequencing Read Length Recommendation

CAUTION

TELL-Read pipeline v1.1 or above is required to analyze sequencing data generated from TELL-Seq™ libraries prepared with TELL Bead Plex and works for data generated from standard TELL Bead as well.

1. Paired-end sequencing is recommended.
2. TELL-Seq™ library Index 1 is 18-base, and Index 2 is 8-base (T-series). There are 26-bases for both TELL-Seq™ indexes compared to Illumina's standard 16-bases for dual indexes. The extra 10-cycle required for sequencing the TELL-Seq™ library index must be evenly deducted from read 1 and read 2 sequencing cycles. Since the Illumina sequencing reagents guarantee 2 extra

cycles, 4-cycles for read 1 and 4-cycles for read 2 need to be deducted. Recommended sequencing lengths are 2×146 PE to 2x150 PE for dual index run.

Sequencing Depth Consideration

Adequate sequencing depth is required to get enough TELL Bead coverage. The more TELL Beads used in library amplification to generate a TELL-Seq™ library, the more sequencing reads will be needed to get the desired sequencing depth. However, the fewer TELL Beads used for library amplification, the lower the library complexity, which may lead to a higher duplication rate of sequencing reads. The amount of TELL Beads used and the TELL-Seq™ library complexity required may depend on the genome size and application.

For targeted sequencing of amplicon sample, at least 200x coverage of the sample is recommended in general.

Library Multiplex Primer Index Sequences (i.e., Index 2 Read), T-series

Library Multiplex Primer	For Sample Sheet NovaSeq v1, MiSeq, HiSeq2000/2500	For Sample Sheet NovaSeq v1.5, Next Seq, MiniSeq, HiSeq3000/4000
T501	TGAACCTT	AAGGTTCA
T502	TGCTAAGT	ACTTAGCA
T503	TGTTCTCT	AGAGAACA
T504	TAAGACAC	GTGTCTTA
T505	CTAATCGA	TCGATTAG
T506	CTAGAACA	TGTTCTAG
T507	TAAGTTCC	GGAACTTA
T508	TAGACCTA	TAGGTCTA
T509	CATCCGAA	TTCGGATG
T510	TTATGAGT	ACTCATAA
T511	AGAGGCGC	GCGCCTCT
T512	TAGCCGCG	CGCGGCTA
T513	ACGAATAA	TTATTCGT
T514	TTCGTAGG	CCTACGAA
T515	GATCTGCT	AGCAGATC
T516	CGCTCCGC	GCGGAGCG
T517	AGGCTATA	TATAGCCT
T518	GCCTCTAT	ATAGAGGC
T519	AGGATAGG	CCTATCCT
T520	TCAGAGCC	GGCTCTGA
T521	CTTCGCCT	AGGCGAAG
T522	TAAGATTA	TAATCTTA
T523	AGTAAGTA	TACTTACT
T524	GACTTCCT	AGGAAGTC