USER GUIDE

Celero EZ™ DNA-Seq

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SYMBOL	MEANING
	Catalog Number
	Consult instructions for use
	Contains sufficient for <n> tests
	Warning
	Important
	Expiration Date
	Temperature limitation
	Optional stopping point
	Note
	Manufacturer

Table of Contents

I. Introduction

A. Overview

Intended Use

Celero EZ DNA-Seq is an end-to-end solution for generation of DNA-Seq libraries derived from ds-DNA. Celero EZ DNA-Seq is intended for Research Use Only and not for use in diagnostic procedures.

Features

Celero EZ DNA-Seq comprises a streamlined workflow with DimerFree® DNA library construction with combinatorial or unique dual index barcoded adaptors available for multiplex sequencing. It includes reagents to perform robust and consistent enzymatic fragmentation.

Also included are reagents for Tecan's novel quantitation method, NuQuant®, for time- and cost-savings in measuring molarity for library pooling prior to multiplex sequencing.

Specifications

Input type:	dsDNA, including ds-cDNA
Input amount:	10 ng–500 ng
# Reactions available:	24, 96 (Automation fill)
Barcodes available:	Up to 384 Metaplex®, or 384 UDI
Sequencing platforms:	Illumina NGS

B. Storage and Stability

Celero EZ DNA-Seq is shipped on dry ice and should be unpacked and inspected immediately upon receipt.

- Store the kit at –20 °C in a freezer without a defrost cycle. Ensure the NuQuant Standard is protected from light.

- NuQuant Buffer may be stored at either –20 °C or 4 °C.

This product has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines are warrantied to perform to specifications for 6 months from the date of shipment. Expiration dates listed on the kit label may be longer than the warranty period. Do not use kits that have passed the expiration date.

C. Warnings and Precautions

1. Unpack and inspect the kits immediately upon receiving. In case of severe kit package damage, no dry ice left in the package or ice pack melted, and/or missing components, please contact Tecan NGS Technical Support immediately. Please provide Tecan Genomics with the kit(s) and/or component(s) part number, and lot number. Do not use damaged components.
2. Follow your institution's safety procedures for working with chemicals and handling of biological samples. Follow good laboratory practices and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary. Changing gloves between handling samples is recommended to avoid contamination of sample or reagents.
3. Consult your institution's environmental waste personnel on proper disposal of unused reagents. Check state and local regulations as they may differ from federal disposal regulations. This material may exhibit characteristics of hazardous waste requiring specific disposal requirements. Institutions should check their country hazardous waste disposal requirements.

4. If appropriate, an SDS for this product is available on the Tecan Genomics website at www.nugen.com/products/celero-ez-dna-seq-library-preparation-kit

D. Before You Start

Please review this User Guide before using this kit for the first time, including the "Components", "Planning the Experiment", "Protocol" and "FAQ" sections.
For more information, visit the Celero EZ DNA-Seq page at www.nugen.com/products/celero-ez-dna-seq-library-preparation-kit

New to NGS? Contact Tecan NGS Technical Support at techserv-gn@tecan.com for tips and tricks on getting started.

A. Reagents Provided

This kit is provided in a 24 (-24) or 96 (-A01) reaction format. A01 fill sizes may be used in manual or automation workflows.

Celero EZ DNA-Seq (Part Nos. 0568 and 0569) is a bundle of library construction core reagents (0362; Table 1) and an adaptor plate (Table 2).

Both Metaplex and UDI barcode options (up to 384) are available for scalability and flexibility.

Table 1. Celero EZ DNA-Seq Core Reagents (Part No. 0362)

COMPONENT	0362-24 PART NUMBER	0362-A01 PART NUMBER	VIAL LABEL
Fragmentation Enzyme V1	S02590	S02591	Blue
Fragmentation Buffer V1	S02592	S02593	Blue
Ligation Mix V1	S02504	S02509	Yellow
Finishing/Amplification Mix V2	S02582	S02583 (2)	Red
NuQuant Standard*	S02512	S02512	Clear
NuQuant Buffer*	S02516	S02517	Clear
DNA Resuspension Buffer Mix (DR1)	S02520	S02303 (2)	Clear
Nuclease-free Water (D1)	S01001	S01113	Green

*NuQuant is enabled for use with Part Nos. 0568 and 0569A-A01 only. For questions or concerns please contact Tecan NGS Technical Support.

Table 2. Metaplex and UDI Adaptor Plates

KIT PART NUMBER	COMPONENT	0568 PART NUMBER	LABEL
0568-24	Metaplex A 24-Plex V1	S02529	Yellow
0568A-A01	Metaplex A 96-Plex V1	S02530	
0568B-A01	Metaplex B 96-Plex V1	S02531	
0568C-A01	Metaplex C 96-Plex V1	S02532	
0568D-A01	Metaplex D 96-Plex V1	S02533	
0569-24	UDI 24-Plex	S02695	
0569A-A01	UDI A 96-Plex	S02534	
0569B-A01	UDI B 96-Plex	S02702	
0569C-A01	UDI C 96-Plex	S02709	
0569D-A01	UDI D 96-Plex	S02710	

Note: The reagents in Celero EZ DNA-Seq are similar to reagents in our other kits; however, unless the component part numbers are identical, these reagents do not have exactly the same composition and, therefore, are not interchangeable. Do not exchange or replace one reagent named, for example, A1 with another A1, as it will adversely affect performance.

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Agilent 2100 Bioanalyzer or 2200 TapeStation Instrument, or other equipment for electrophoretic analysis of nucleic acids
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - Microcentrifuge for 0.2 mL tube strips or plates
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
  - 2–20 µL or 5–50 µL multichannel pipette and 20–200 µL or 20–300 µL multichannel pipette for sample mixing
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block, adjustable heated lid, and 100 µL reaction capacity
  - Nanodrop® Spectrophotometer for assessment of input DNA purity
  - Qubit® 2.0, 3.0 or 4 Fluorometer (Thermo Fisher Scientific) or appropriate fluorometer and accessories for quantification of fragmented DNA and amplified libraries.

- **Reagents**
  - Agencourt® RNAClean XP Beads or AMPure XP Beads (Beckman Coulter, Cat. #A63987 or A63881)
  - Ethanol, Absolute (200 Proof), Molecular Biology Grade (Fisher Scientific Cat. #BP2818), for purification steps
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Fisher Scientific, Cat. #75793), for diluting nucleic acids
  - Nuclease-free water (Fisher Scientific, Cat. #BP2484), for diluting nucleic acids
  - Agilent High Sensitivity DNA Kit (Agilent, Cat. #5067-4626) or equivalent
  - EvaGreen®, 20X (Biotium, Cat. #31000) optional; for optimizing Library Amplification with qPCR
- **Supplies and Labware**
  - Barrier (filter) pipette tips, nuclease-free
  - Low-retention 1.5 mL and 0.5 mL microcentrifuge tubes, nuclease-free
  - 0.2 mL PCR strip tubes or 0.2 mL thin-wall PCR plates
  - Magnetic stand for 0.2 mL strip tubes or plates. (Beckman Coulter Cat. #A29164 or A32782; Thermo Fisher Scientific Cat. #12331D, 12027, or 12332D; Promega Cat. #V8351). Other magnetic stands may be used as well, although their performance has not been validated by Tecan.
  - Thin-wall, clear, 0.5 mL PCR tubes (Axygen® PCR-05-C tubes (VWR International, Cat. #10011- 830), for NuQuant assay with Qubit
  - Cleaning solutions such as RNaseZap® RNase Decontamination Solution (Thermo Fisher Scientific, Cat. #AM9780) and DNA-OFF™ (MP Biomedicals, Cat. #11QD0500)
  - 96-well plate sealing foil (Thermo Fisher Scientific, Cat. #AB1720)
  - Disposable gloves
  - Kimwipes
  - Ice bucket

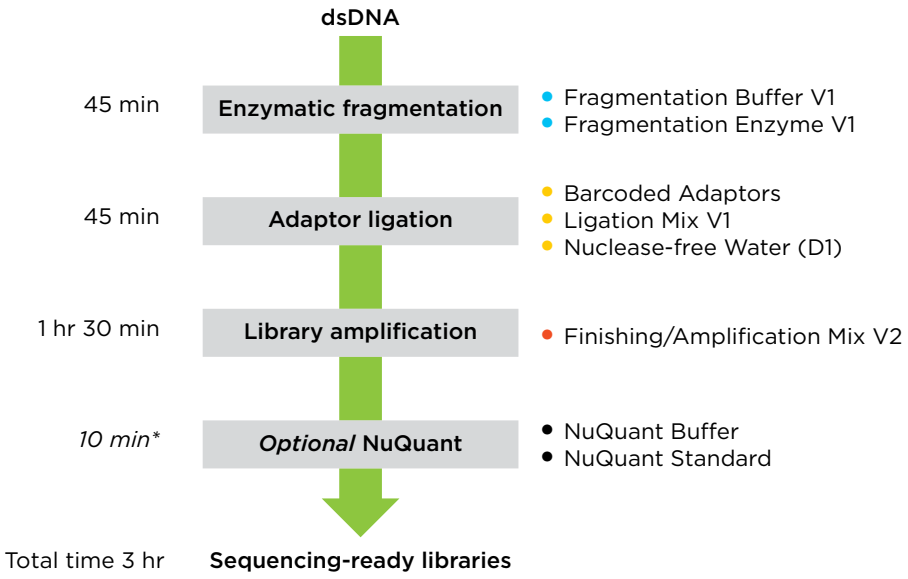
To Order:

- Agilent, www.agilent.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, www.biotium.com
- Fisher Scientific, www.fishersci.com
- MP Biomedicals, www.mpbio.com
- Promega, www.promega.com
- Thermo Fisher Scientific, www.thermofisher.com
- VWR International, www.vwr.com

A. Workflow and Time Required

The streamlined Celero EZ DNA-Seq workflow starts with enzymatic fragmentation of intact DNA followed by adaptor ligation and PCR amplification to produce the final library (Figure 1). The entire workflow can be completed in as few as 3 hours and yields DNA libraries ready for sequencing on Illumina platforms.

Figure 1. Celero EZ DNA-Seq Workflow



*Estimated time for NuQuant on Qubit using the Celero PCR app. For NuQuant instructions on the plate reader please contact Tecan NGS Technical Support.

B. Input DNA Requirements

DNA Quantity

DNA input must be between 10 - 500 ng of genomic DNA or ds-cDNA. We strongly recommend accurate quantification of DNA to ensure the minimum input requirement is met.

DNA Purity

DNA samples must be free of contaminating proteins, RNA, and other cellular material, organic solvents (including phenol and ethanol), and salts used in many nucleic acid isolation methods. If using a DNA isolation method based on organic solvents, we recommend column purification after isolation.

One measure of DNA purity is the ratio of absorbance readings. The A260:A280 ratio for DNA samples should be greater than 1.8 and A260:A230 should be greater than 2.0. Use of DNA samples with lower ratios may result in low yield.

DNA Integrity

This kit is designed for use with DNA samples of high molecular weight with little or no evidence of degradation. This product has not been validated for use with degraded or FFPE DNA.

C. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes and equipped with an adjustable heated lid. Prepare the programs shown in Table 3 following the operating instructions provided by the manufacturer.

Important: Program 1 requires a heated lid setting of 65 °C.

Table 3. Thermal Cycler Programming

ENZYMATIC FRAGMENTATION		VOLUME
Program 1 Enzymatic Fragmentation	Ensure block is pre-warmed prior to incubating samples. Set heated lid to 65 °C. 25 °C – X min, 55 °C – 10 min, hold at 4 °C	15 µL
LIGATION		**VOLUME**
Program 2 Adaptor Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 10 °C	30 µL
AMPLIFICATION		**VOLUME**
Program 3 Library Amplification	72 °C – 2 min, 95 °C – 3 min, N(98 °C – 20 s, 65 °C – 30 s, 72 °C – 1 min), 72 °C – 1 min, hold at 10 °C	100 µL

Important

- The fragmentation time (X) should be adjusted based on the desired fragment length. Refer to Table 5 for specific guidelines.
- The number of cycles used for Library Amplification (N) depends on the starting amount of DNA. An optional real-time PCR protocol is included in **Appendix B**. for determining the appropriate number of PCR cycles. For more information, contact Tecan NGS Technical Support.

D. Selecting Appropriate Fragment Size

Celero EZ DNA-Seq provides robust, reproducible, tunable fragmentation. Conditions for generation of different insert sizes are described in Table 5 on page 14. Selection of the appropriate insert size will depend upon the experimental goals, and sequencing configuration should be considered in order to optimize the use of sequencing reads.

Two bead purification options are provided after library amplification for further optimization of library fragment size: a standard bead purification, and a double-sided bead purification (double-size selection) for tighter insert distributions. The double-sided purification will reduce final library yield. Do not over cycle as improper size selection may result. General guidelines for PCR cycle number are provided in Table 7.

E. Working with the 24- and 96-Plex Adaptor Plates

The Adaptor Plate included with the 24- and 96-reaction Celero EZ DNA-Seq kits contain dual index adaptor mixes with eight-base barcodes. Each well contains a sufficient volume of adaptor mix for preparation of a single library. The adaptor plates are sealed with a foil seal designed to provide airtight storage.

Thaw adaptor plate on ice and spin down. Do NOT warm above room temperature. Make sure all adaptor mixes are collected at the bottom of the wells and place the adaptor plate on ice after centrifugation. When working with the adaptor mixes, puncture the seal for each well you wish to use with a fresh pipet tip, and transfer the entire 15 µL of sample into each well. Mix well by pipetting, and transfer the reactions into PCR tubes. The remaining wells of the plate should remain sealed for use at a later date. Cover used wells with a new foil seal to prevent any remaining adaptor-containing liquid from contaminating future reactions.

For details regarding barcode sequences, please see **Appendix A** on page 22.

F. Bead Purifications

Agencourt Beads

Ampure XP or RNA Clean XP Beads (Agencourt beads) are suitable for use with this kit. There are modifications to the Agencourt beads' standard procedure; therefore, you must follow the protocols outlined in this user guide for the use of these beads.

- Remove beads from 4 °C and leave at room temperature for at least 30 minutes prior to use. Cold beads and buffer will result in reduced recovery.
- Prior to use, ensure beads are fully resuspended by vortexing or inverting and tapping the tube.
- If using strip tubes or partial plates, ensure they are firmly placed on the magnetic plate. The use of individual tubes is not advised as they are not very stable on the magnetic plates.
- It is critical to let the beads separate on the magnet for the full time indicated at each step. Removing the binding buffer before the beads have completely separated will impact yields.
- After the binding step has been completed, take care to minimize bead loss when removing the binding buffer. Loss of beads at this step may impact yields. With the samples placed on the magnetic plate, carefully remove the specified quantity of binding buffer from each sample to avoid disturbing the beads.
- Ensure that the 70% ethanol wash is freshly prepared from fresh ethanol stocks. Lower percent ethanol mixes will reduce recovery.
- It is critical that all residual ethanol be removed prior to elution. Therefore, when removing the final ethanol wash, first remove most of the ethanol, then allow the excess to collect at the bottom of the tube before removing the remaining ethanol. This also reduces the required bead air-drying time.
- After drying the beads, inspect each tube carefully and make certain that all the ethanol has evaporated before proceeding with the amplification step.

Preparation of EtOH Wash Solution

Prepare a 70% EtOH wash solution by combining 100% EtOH and nuclease-free water. Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes.

This protocol requires 0.4 mL of 70% EtOH solution per sample.

Important:

- It is critical that the ethanol solution in the purification steps be prepared fresh on the same day of the experiment from a recently opened stock container.
- Measure both the ethanol and the water components carefully prior to mixing. Failure to do so can result in a higher than anticipated aqueous content, which may reduce amplification yield.

1X REACTION VOLUME*	100% EtOH	NUCLEASE-FREE WATER
0.40 mL	0.28 mL	0.12 mL

*A minimum of 10% extra volume should be prepared for each sample.

G. NuQuant

NuQuant® is a novel method to accurately measure molar concentrations of NGS libraries without the need for separate fragment size analysis. The library molar concentration can be directly measured using fluorometers or standard plate readers, then pooled and quantified for sequencing.

NuQuant is compatible with Qubit 2.0, 3.0 or 4 as well as a wide variety of fluorescent plate readers.

For Qubit-based quantification, an app is required. The apps and installation instructions are available on GitHub: https://nugentechnologies.github.io/NuQuant/

Preparation of Diluted NuQuant Standard

Celero EZ DNA-Seq includes a 50X NuQuant Standard stock solution. The fluorescence of this stock corresponds to a 21.5 µM library generated with the kit. This stock solution should be diluted prior to use on fluorometers or fluorescent plate readers following the protocol below. The 1X NuQuant Standard corresponds to a 430 nM Celero EZ DNA-Seq library.

Note:

- NuQuant Standards should be protected from light.
- NuQuant Standard diluted to 1X for Qubit may be stored at 2–8 °C for up to two months. Do not freeze the 1X NuQuant Standard.

1. Remove concentrated 50X NuQuant Standard stock solution and thaw on ice. Mix by vortexing, spin down and place on ice.
2. Prepare diluted NuQuant Standard in a low-retention microcentrifuge tube.

a. For use with Qubit, combine 2 µL of 50X NuQuant Standard stock solution and 98 µL of NuQuant Buffer. Mix thoroughly by vortexing, spin down and store at 2–8 °C.

b. For use with fluorescent plate readers, make a fresh dilution of the 50X NuQuant Standard to 1X with Low-EDTA TE Buffer, 1X, pH 8.0. Mix thoroughly by vortexing and spin down.

NuQuant Application

NuQuant is compatible with Qubit 2.0, 3.0 or 4. An app is required. The apps and installation instructions are available on GitHub: https://nugentechnologies.github.io/NuQuant/

For other fluorometers, a standard curve must be prepared. For more information, contact Tecan NGS Technical Support at techserv-gn@tecan.com.

Important: NuQuant quantitation must be performed prior to storage at –20 °C.

H. Sequencing Recommendations and Guidelines

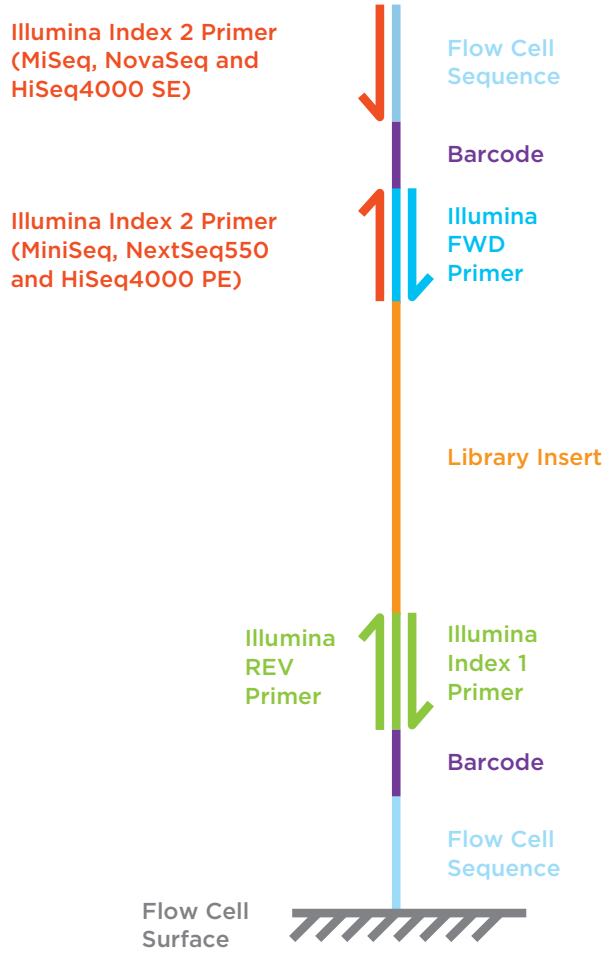
Celero EZ DNA-Seq produces libraries compatible with Illumina NGS platforms. These libraries should be sequenced using the Illumina protocol for multiplex sequencing, following the recommendations for the specific sequencer.

For sequencing recommendations and questions contact Tecan NGS Technical Support at techserv-gn@tecan.com.

Index Read Recommendations

Celero EZ DNA-Seq libraries contain 8 base Metaplex or Unique Dual Index barcodes for sample multiplexing. These barcodes differ from the sequences used by Illumina and can be found in Appendix A.

Figure 2. Celero EZ DNA-Seq Library Structure.



I. Data Analysis

For Celero EZ DNA-Seq libraries, follow the recommendations in the Illumina technical support documentation on parsing barcodes. The sequences of the Celero EZ DNA-Seq barcodes will need to be entered prior to parsing. These sequences are found in Appendix A.

Once the data have been parsed according to sample, additional sample specific data analysis may be employed according to the requirements of the experiment.

J. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

Protocol Notes

Controls

- We recommend the routine use of a positive control DNA, especially the first time a reaction is set up. The use of a positive control DNA will establish a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.
- Routine use of a no template control (NTC) is recommended to monitor the work environment for potential carryover contamination of previous libraries.

General Workflow

- Set up no fewer than 4 reactions at a time for 24 reaction kits and 8 reactions at a time for 96 reaction kits to ensure that you are not pipetting very small volumes and to ensure sufficient reagent recoveries for the full nominal number of samples from the kit. Making master mixes for fewer than 8 samples at a time may affect reagent recovery volumes.
- Thaw components used in each step and immediately place them on ice. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed. Do not thaw all reagents at once.
- Do not vortex or warm any reagents or adaptors in this kit unless otherwise directed.
- When preparing master mixes, use the minimal amount of extra material to ensure you are able to run the maximum number of reactions using the components provided in the kit.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer from the pipet tip to the reaction mix.
- When instructed to mix via pipetting, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.

Reagents

- Use the nuclease-free water provided with the kit or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Components and reagents from other Tecan products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol for washes in the purification protocols.
- Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

This protocol includes workflows for DNA-Seq library construction followed by a standard or double-sided bead purification. For double-sided bead purifications, follow sections **A. Sample Preparation** through **D. Library Amplification**, then continue to **Appendix C**.

For each section of the protocol, remove reagents from recommended storage conditions listed on page 1. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

Return all reagents to their appropriate storage conditions promptly after use unless otherwise instructed.

A. Sample Preparation

1. Aliquot 10–500 ng input DNA in a 0.2 mL tube strip or plate.
2. Bring volume up to 10 µL with low-EDTA TE or nuclease-free water.

B. DNA Fragmentation

1. Remove nuclease-free water (Green: D1) from –20 °C storage and place at room temperature for use in the next section of the protocol.

Table 4. Fragmentation Master Mix

REAGENT	FRAGMENTATION BUFFER V1 (BLUE)	FRAGMENTATION ENZYME V1 (BLUE)
1X REACTION VOLUME	3 µL	2 µL

Note:
- Set up all enzymatic fragmentation reactions on ice.
- Vortexing the Fragmentation Enzyme and Fragmentation Master Mix is critical to ensure consistent fragmentation.

1. Thaw Fragmentation Buffer V1 and Fragmentation Enzyme V1 on ice. Mix by vortexing, spin down and place on ice.
2. Prepare Fragmentation Master Mix by combining Fragmentation Buffer and Fragmentation Enzyme in an appropriately sized capped tube according to the volumes shown in Table 4. Mix well by vortexing, spin down and place on ice.
3. Add 5 µL of Fragmentation Master Mix to each sample for a total of 15 µL. Mix well by vortexing, spin down and place on ice.

Note: After addition of Fragmentation Master Mix to the sample, vortex thoroughly.

4. Place the tubes in a pre-warmed thermal cycler with the heated lid set to 65 °C to run Program 1 (Enzymatic Fragmentation; see Table 3). Please see Table 5 for fragmentation guidelines (X).

   25 °C – X min, 55 °C – 10 min, hold at 4 °C

   Important: Program 1 requires a heated lid setting of 65 °C.

Table 5. Fragmentation Guidelines

TARGET INSERT SIZE	FRAGMENTATION TIME (X)
200 bp	30 min
300 bp	20 min
400–500 bp	10 min

5. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.

C. Adaptor Ligation

1. Remove the Adaptor Plate from –20 °C storage and thaw on ice. Spin down and return to ice.

Table 6. Ligation Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	LIGATION MIX V1 (YELLOW)
1X REACTION VOLUME	2.25 µL	6.75 µL

2. Mix Ligation Mix V1 by pipetting, spin down and place on ice.
3. Puncture the required number of wells on the Adaptor Plate with a fresh pipette and transfer the entire 15 µL of each sample into the appropriate well. Mix thoroughly by pipetting and transfer the entire reaction back into a PCR tube strip or plate.

   Note: All samples intended to share the same sequencing flow cell lane should have unique barcoded adaptors.

4. Prepare a Ligation Master Mix by combining Ligation Mix and D1 in an appropriately sized capped tube according to the volumes shown in Table 6. Mix well by pipetting, spin down and place on ice.

   Note: The Ligation Master Mix is very viscous. Please be sure to pipet this reagent slowly.

5. Add 9 µL of Ligation Master Mix to each sample for a total of 30 µL. Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.
6. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (Adaptor Ligation; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 10 °C
7. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.

D. Library Amplification

1. Remove Agencourt beads and D1 from storage and place on the benchtop to reach room temperature for use in the next section of the protocol.
2. Thaw Finishing/Amplification Mix V2 on ice. Mix by pipetting, spin down and place on ice.
3. Add 70 µL of Finishing/Amplification Mix V2 to each sample for a total of 100 µL. Mix by pipetting, spin down and place on ice.
4. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (Library Amplification; see Table 3). Please see Table 7 for PCR cycling guidelines (N).

   72 °C – 2 min, 95 °C – 3 min, N(98 °C – 20 s, 65 °C – 30 s, 72 °C – 30 s), 72 °C – 1 min, hold at 4 °C

Table 7. Recommended PCR Cycles for Amplification

STARTING INPUT (ng)	PCR CYCLES (N)
10-100	6-9
100-500	4-6

Note:
- The cycle numbers provided are recommended starting points. The precise number of PCR cycles required (N) depends on a number of factors including sample type, quality and input amount, and may be decreased or increased based on the requirements for a given sample. See **Appendix B** to determine the appropriate number of PCR cycles.
- A minimum of 4 cycles is required to use NuQuant.

5. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Continue to section **E. Standard Library Purification** for library purification. For double-sided library purification, see **Appendix C**.

E. Standard Library Purification

1. Remove NuQuant reagents from storage for use in the next section:
   - Remove diluted NuQuant Standard from storage. Mix thoroughly by vortexing, spin and place at room temperature. Protect from light.
   - Remove NuQuant Buffer from storage and thaw at room temperature. Mix thoroughly by vortexing, spin and place on benchtop.
2. Ensure the Agencourt beads and D1 have completely reached room temperature before proceeding.
3. Prepare a 70% ethanol wash solution.
4. Resuspend the beads by vortexing. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.
5. Add the appropriate volume of bead suspension to each sample according to the volumes given in Table 8 . Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the total sample volume.†

Table 8. Bead Volume for Standard Purification

INSERT SIZE	AGENCOURT BEADS (RATIO)
200 bp	80 µL (0.8X)
300 bp	70 µL (0.7X)
400–500 bp	60 µL (0.6X)

6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand at least 5 minutes to completely clear the solution of beads.
8. Carefully remove and discard the supernatant, taking care not to disturb the beads.

   Important: It is critical to remove as much of the supernatant as possible. Use at least two pipetting steps and allow excess buffer to collect at the bottom of the tubes after removing most of the supernatant in the first pipetting step.
9. Remove the tubes from the magnet.
10. Add 50 µL of DNA Resuspension Buffer (DR1) to each sample and pipet to completely resuspend the beads.
11. Add the appropriate volume of bead suspension to each sample according to the volumes given in Table 9. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the total sample volume.

† If tubes have become noticeably warped or plastic integrity compromised, transfer entire sample to fresh tubes.

Table 9. Bead Volume for Standard Purification

INSERT SIZE	AGENCOURT BEADS (RATIO)
200 bp	40 µL (0.8X)
300 bp	35 µL (0.7X)
400–500 bp	30 µL (0.6X)

12. Incubate at room temperature for 10 minutes.
13. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
14. Carefully remove and discard the supernatant, taking care not to disturb the beads.

    Important: It is critical to remove as much of the supernatant as possible. Use at least two pipetting steps and allow excess buffer to collect at the bottom of the tubes after removing most of the supernatant in the first pipetting step.

15. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
16. Remove the 70% ethanol wash using a pipette.
17. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Important: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

18. Air dry the beads on the magnet for 3 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
19. Remove the tubes from the magnet.
20. Add 20 µL room temperature D1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
21. Transfer the tubes to the magnet and let stand for 3 minutes to completely clear the solution of beads.
22. Carefully remove 20 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.

F. Quantitative and Qualitative Assessment of the Library

Library Quantification with NuQuant†

Note: A detailed protocol for the NuQuant Qubit assay is provided below. For information on using NuQuant with other fluorometers and fluorescent plate readers, please contact Tecan NGS Technical Support.

1. Ensure NuQuant Buffer and diluted NuQuant Standard (prepared in section III. G.) have reached room temperature before proceeding.
2. Aliquot 200 µL NuQuant Buffer into a new thin-wall, clear, 0.5 mL tube. Label this tube S1.
3. Aliquot 195 µL NuQuant Buffer into thin-wall, clear, 0.5-mL tubes. Prepare one tube for the diluted standard and one tube for each library. Label the diluted standard tube S2.

Note: The S2 Standard represents a Celero EZ DNA-Seq with NuQuant library with a concentration of 10.7 nM.

4. Add 5 µL of diluted NuQuant Standard or library to each tube containing NuQuant Buffer from Step 3.
5. Close lids securely, mix thoroughly by vortexing, and spin briefly to collect all liquid into the bottom of the tube.

Important:
- The diluted NuQuant Standard may settle over time. Mix thoroughly before aliquoting.
- If standards and libraries are not thoroughly mixed, inaccurate results may occur.

6. Measure samples as directed for your specific quantification platform:
   a. Qubit 2.0: Select "Celero PCR" from the Qubit home screen. Follow the on screen prompts to read S1, S2, and samples.

Note: For Qubit 2.0, the "Assay Concentration" is displayed after reading each sample. To calculate the stock concentration of each library, select the "Calculate Stock Conc." button, and change the "Volume of Sample Used" to 5 µL. The correct stock concentration of your library will be displayed.

   b. Qubit 3.0 and 4: Navigate to the saved location of the NuQuant app and select "Celero PCR". Follow the on screen prompts to read S1, S2, and samples.

Note: For Qubit 3.0 and 4, enter the original sample volume (5 µL) after reading standard 2. The result for each sample will be displayed as the "Original Calculated Sample Concentration" and the "Qubit Tube Concentration."

Library Pooling and Assessment

1. Pool the libraries according to NuQuant concentration. An example using the calculation V1=(C2/N*V2)/C1 , where C = concentration, N = number of libraries in the pool and V = volume, is provided in Table 10 below.

† Note: For quantification of libraries without use of NuQuant, a qPCR-based method is recommended. Contact Tecan NGS Technical Support for more information.

Table 10. Example Library Pooling Calculation using NuQuant Concentration

LIBRARY	NUQUANT CONCENTRATION (C_1)	TARGET CONCENTRATION OF POOL (C_2)	TARGET VOLUME OF POOL (V_2)	VOLUME OF LIBRARY TO ADD TO POOL (V_1)
1	90 nM	10 nM	100 µL	11.1 µL
2	95 nM			10.5 µL
3	85 nM			11.8 µL
4	90 nM			11.1 µL
			Volume of low-EDTA or nuclease-free water to add	55.5 µL
			Total volume of library pool	100 µL

2. Validate the library pool for sequencer loading following the Illumina guidelines, "Best practices for manually normalizing library concentrations" for your specific sequencer. See **Appendix A**. of this guide for guidelines on color balancing and multiplexing of Tecan libraries. A typical fragment distribution for Celero EZ DNA-Seq libraries prepared with high-quality input DNA is shown in Figure 3 and 4.

Important: As with any library quantitation method, individual operators, lab equipment and instruments will influence the optimal loading quantitation. Validation of the library pool should be determined empirically for each new library type and sequencer platform.

Figure 3. Fragment distribution of an amplified library of insert sizes 200, 300, and 500 base pairs constructed from 10 ng input DNA with a single side bead purification, on a DNF-474 High Sensitivity NGS Fragment Analysis chip. Adaptors add 136 bp to the library.

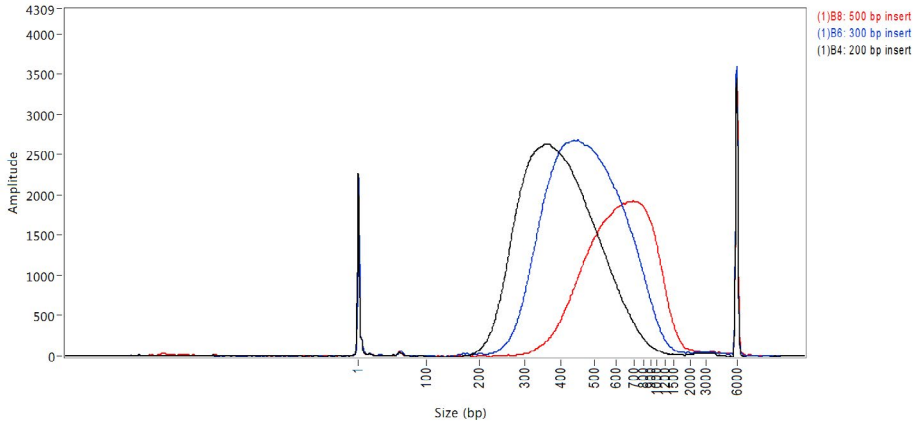
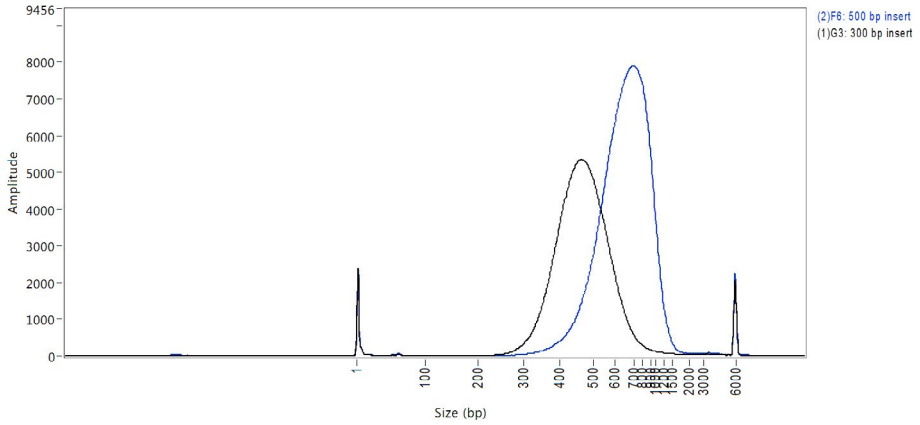


Figure 4. Fragment distribution of an amplified library of insert sizes 300 and 500 base pairs constructed from 10 ng input DNA with a double sided bead purification, on a DNF-474 High Sensitivity NGS Fragment Analysis chip.



3. Prepare libraries for sequencing following the Illumina "Denature and Dilute Libraries Guide" for your specific sequencer.

V. Technical Support

For help with any of our products, please contact Tecan NGS Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only) or email techserv-gn@tecan.com.

In Europe contact Tecan NGS Technical Support at +31.13.5780215 (phone) or email europe-gn@tecan.com.

In all other locations, contact your Tecan NGS reagent distributor for technical support.

A. Barcode Sequences and Guidelines for Multiplex Experiments

Index 1 (i7) barcode sequences for the 24- and 96-plex Metaplex Adaptor Plates are given in Table 11, with barcodes for the 24-plex plate given in positions A01-H03. Barcodes are color balanced in pairs, and in sets of 8 by column.

Index 2 (i5) sequences for Metaplex Adaptor Plates are given in Table 12.

Barcodes for UDI A are given in Table 13. Barcodes for the 24-plex UDI plate are given in positions A01-H03. UDI plates B-D are also available. All UDI barcode sequences are color balanced in sets of 8 by column.

Text versions of all barcode sequences can be found at https://tecangenomics.github.io, or contact Tecan NGS Technical Support.

Table 11. Barcode sequences for Index 1 (i7).

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CGCTACAT	A05	AGGTTCCT	A09	GCCTTAAC
B01	AATCCAGC	B05	GAACCTTC	B09	ATTCCGCT
C01	CGTCTAAC	C05	AAGTCCTC	C09	ATCGTGGT
D01	AACTCGGA	D05	CCACAACA	D09	GCTACAAC
E01	GTCGAGAA	E05	ATAACGCC	E09	TCTACGCA
F01	ACAACAGC	F05	CCGGAATA	F09	CTCCAATC
G01	ATGACAGG	G05	CCAAGTAG	G09	ACTCTCCA
H01	GCACACAA	H05	AAGGACCA	H09	GTCTCATC
A02	CTCCTAGT	A06	ACGCTTCT	A10	GCCAGAAT
B02	TCTTCGAC	B06	CTATCCAC	B10	AATGACGC
C02	GACTACGA	C06	TGACAACC	C10	GTACCACA
D02	ACTCCTAC	D06	CAGTGCTT	D10	ACGATCAG
E02	CTTCCTTC	E06	TCACTCGA	E10	TAACGTCG
F02	ACCATCCT	F06	CTGACTAC	F10	CGCAACTA
G02	CGTCCATT	G06	GTGATCCA	G10	AACACTGG
H02	AACTTGCC	H06	ACAGCAAG	H10	CCTGTCAA
A03	GTACACCT	A07	TGCTGTGA	A11	TCCTGGTA
B03	ACGAGAAC	B07	CAACACAG	B11	CATCAACC
C03	CGACCTAA	C07	CCACATTG	C11	AGCAGACA
D03	TACATCGG	D07	TAGTGCCA	D11	GAAGACTG
E03	ATCGTCTC	E07	TCGTGCAT	E11	TCTAGTCC
F03	CCAACACT	F07	CTACATCC	F11	CTCGACTT
G03	TCTAGGAG	G07	CATACGGA	G11	CTAGCTCA
H03	CTCGAACA	H07	TGCGTAAC	H11	TCCAACTG
A04	ACGGACTT	A08	CAGGTTCA	A12	GACATCTC
B04	CTAAGACC	B08	AGAACCAG	B12	ACTGCACT
C04	AACCGAAC	C08	GAATGGCA	C12	GTTCCATG
D04	CCTTAGGT	D08	AGGCAATG	D12	ACCAAGCA
E04	CCTATACC	E08	TAGGAGCT	E12	CTCTCAGA
F04	AACGCCTT	F08	CGAACAAC	F12	ACTCTGAG
G04	TCCATTGC	G08	CATTCGTC	G12	GCTCAGTT
H04	CAAGCCAA	H08	AGCCAACT	H12	ATCTGACC

Table 12. Metaplex barcode sequences for Index 2 (i5)

PART NUMBER	BARCODE SEQUENCE
0568A-A01	AACCTACG
0568B-A01	GCATCCTA
0568C-A01	CAACGAGT
0568D-A01	TGCAAGAC

Table 13. Barcode sequences for Unique Dual Index plate A.

PLATE LOCATION	INDEX SEQUENCE	INDEX 2 SEQUENCE	PLATE POSITION	INDEX SEQUENCE	INDEX 2 SEQUENCE	PLATE POSITION	INDEX SEQUENCE	INDEX 2 SEQUENCE
A01	CGCTACAT	AACCTACG	A05	AGGTTCCT	TCGAACCT	A09	GCCTTAAC	CCGTTATG
B01	AATCCAGC	GCATCCTA	B05	GAACCTTC	CAAGGTAC	B09	ATTCCGCT	TGTCGACT
C01	CGTCTAAC	CAACGAGT	C05	AAGTCCTC	AGCTACCA	C09	ATCGTGGT	CTCTATCG
D01	AACTCGGA	TGCAAGAC	D05	CCACAACA	CATCCAAG	D09	GCTACAAC	ACTGCTTG
E01	GTCGAGAA	CTTACAGC	E05	ATAACGCC	CTCACCAA	E09	TCTACGCA	CGCCTTAT
F01	ACAACAGC	ACCGACAA	F05	CCGGAATA	TCAGTAGG	F09	CTCCAATC	ATAGGTCC
G01	ATGACAGG	ACATGCCA	G05	CCAAGTAG	GAACGTGA	G09	ACTCTCCA	TGATCACG
H01	GCACACAA	GAGCAATC	H05	AAGGACCA	AGGAACAC	H09	GTCTCATC	CGGATCAA
A02	CTCCTAGT	CCTCATCT	A06	ACGCTTCT	CCTAAGTC	A10	GCCAGAAT	TACTAGCG
B02	TCTTCGAC	TACTGCTC	B06	CTATCCAC	AACGCACA	B10	AATGACGC	TGGACCAT
C02	GACTACGA	TTACCGAC	C06	TGACAACC	GTCAACAG	C10	GTACCACA	GCGCATAT
D02	ACTCCTAC	CCGTAACT	D06	CAGTGCTT	ACACCTCA	D10	ACGATCAG	ATCGCAAC
E02	CTTCCTTC	TTCCAGGT	E06	TCACTCGA	TATGGCAC	E10	TAACGTCG	TCAGCCTT
F02	ACCATCCT	CCATGAAC	F06	CTGACTAC	CGCAATGT	F10	CGCAACTA	CATTGACG
G02	CGTCCATT	TTCCTCCT	G06	GTGATCCA	ACTCAACG	G10	AACACTGG	ACAGGCAT
H02	AACTTGCC	CCAACTTC	H06	ACAGCAAG	GTCTGCAA	H10	CCTGTCAA	AGGTCTGT
A03	GTACACCT	GAGACCAA	A07	TGCTGTGA	CACGATTC	A11	TCCTGGTA	CAGATCCT
B03	ACGAGAAC	ACAGTTCG	B07	CAACACAG	AGAAGCCT	B11	CATCAACC	CTCCTGAA
C03	CGACCTAA	CTAACCTG	C07	CCACATTG	TACTCCAG	C11	AGCAGACA	AGAGGATG
D03	TACATCGG	TCCGATCA	D07	TAGTGCCA	CGTCAAGA	D11	GAAGACTG	CACCATGA
E03	ATCGTCTC	AGAAGGAC	E07	TCGTGCAT	CTGTACCA	E11	TCTAGTCC	CGGTAATC
F03	CCAACACT	GACGAACT	F07	CTACATCC	TCACCTAG	F11	CTCGACTT	GAGTGTGT
G03	TCTAGGAG	TTGCAACG	G07	CATACGGA	AACACCAC	G11	CTAGCTCA	AACTGAGG
H03	CTCGAACA	CCAACGAA	H07	TGCGTAAC	CGTCTTCA	H11	TCCAACTG	TGTGTCAG
A04	ACGGACTT	ATCGGAGA	A08	CAGGTTCA	AACGTAGC	A12	GACATCTC	TGTCACAC
B04	CTAAGACC	CCTAACAG	B08	AGAACCAG	GCAACCAT	B12	ACTGCACT	AGATCGTC
C04	AACCGAAC	CATACTCG	C08	GAATGGCA	GATCCACT	C12	GTTCCATG	CAATGCGA
D04	CCTTAGGT	TGCCTCAA	D08	AGGCAATG	ACCTAGAC	D12	ACCAAGCA	TGCTTGCT
E04	CCTATACC	TACAGAGC	E08	TAGGAGCT	CTAGCAGT	E12	CTCTCAGA	AATGGTCG
F04	AACGCCTT	CGAGAGAA	F08	CGAACAAC	TCGATGAC	F12	ACTCTGAG	AGTTGTGC
G04	TCCATTGC	AGGTAGGA	G08	CATTCGTC	TTGGTGCA	G12	GCTCAGTT	GTATCGAG
H04	CAAGCCAA	GAACGAAG	H08	AGCCAACT	AGTGCATC	H12	ATCTGACC	GTACGATC

For UDI barcodes B-D and text versions of all barcode sequences visit https://tecangenomics.github.io/

Note: Manual demultiplexing of sequencing data generated on the Illumina MiniSeq, NextSeq, NovaSeq or HiSeq 4000 (PE runs only) instruments may require inputting the reverse complement of the Index 2 (i5) sequences.

B. Library Amplification Optimization with qPCR

When using the kit for the first time, or working with a new sample type or input amount, we recommend performing a qPCR step prior to Library Amplification to determine the optimum number of cycles needed and ensure there is no excess amplification.

Part I: Perform qPCR

Perform a 1/10th scale qPCR reaction as follows:

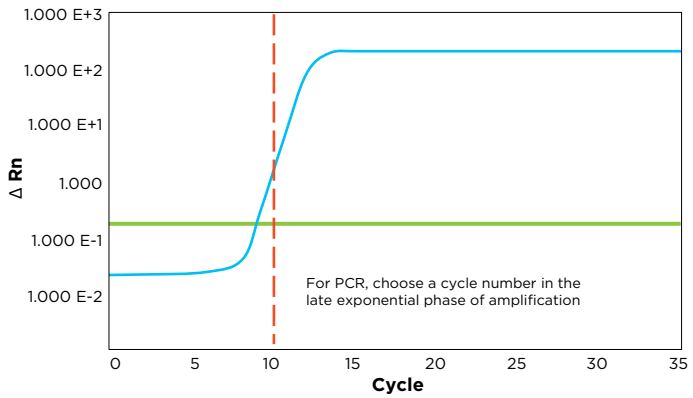
Table 14. Library Amplification qPCR Master Mix

REAGENT	FINISHING/AMPLIFICATION MIX V2 (RED)	20X EVAGREEN
1X REACTION VOLUME	6.5 µL	0.5 µL

1. Prepare a qPCR master mix according to the volumes shown in Table 14.
2. Aliquot 7.0 µL of qPCR master mix per sample into a 0.2 mL qPCR strip or plate.
3. Add 3.0 µL of finished Adaptor Ligation reaction for a total qPCR volume of 10 µL. Mix well by pipetting, spin and place on ice.
4. Perform real-time qPCR with the following cycling conditions:

   72 °C – 2 min, 95 °C – 3 min, 35 cycles (98 °C – 20 s, 65 °C – 30 s, 72 °C – 1 min), 72 °C – 1 min, hold at 10 °C
5. The cycle number used for subsequent library amplification should be within the exponential phase of the amplification plot (10 cycles in Figure 5 as an example).

Figure 5. Stylized qPCR amplification plot.



To amplify the remaining 27 µL of adaptor ligation reaction, prepare a 9/10th reaction as given below.

Part II: Perform Library Amplification

1. Remove Agencourt beads from 4 °C storage and place on the bench top to reach room temperature for use in the next step.
2. Thaw Finishing/Amplification Mix v2 on ice. Mix by pipetting, spin and place on ice.
3. Add 63 µL of Finishing/Amplification Mix v2 to each sample for a total of 90 µL. Mix by pipetting, spin and place on ice.
4. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (Library Amplification; see Table 3.):

   72 °C – 2 min, 95 °C – 3 min, N(98 °C – 20 s, 65 °C – 30 s, 72 °C – 1 min), 72 °C – 1 min, hold at 10 °C
5. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.
6. Add 10 µL of nuclease-free water to each sample. Mix well by pipetting, spin and place on ice.
7. Continue to **Section IV. E**. **for Standard Library Purification**. For double-sided purification, see **Appendix C**.

C. Double-Sided Bead Purification

Included below are two double-sided bead purification protocols designed to achieve 300 bp or 500 bp insert sizes, respectively. These protocols include a two-step bead cut, designed to create a tighter insert distribution. This double bead cut will reduce library yield.

300 bp Size Selection Protocol

1. If using NuQuant:
   a. Remove diluted NuQuant Standard from 4 °C storage. Mix thoroughly by vortexing, spin and place at room temperature. Protect from light.
   b. Remove NuQuant Buffer from –20 °C and thaw at room temperature. Mix thoroughly by vortexing, spin and place on benchtop.
2. Ensure the Agencourt beads have completely reached room temperature before proceeding.
3. Resuspend the beads by vortexing. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.
4. At room temperature, add 70 µL (0.7X vol) of Agencourt beads to 100 µL of library product from **Section IV. D.**

   Important: Accurate pipetting of bead volume is critical. Avoid carrying excess bead volume from outside of the pipet tip to the sample.
5. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the sample volume.†

† If tubes have become noticeably warped or plastic integrity compromised, transfer entire sample to fresh tubes.

6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove and discard the supernatant, taking care not to disturb the beads.

   Important: It is critical to remove as much of the supernatant as possible. Use at least two pipetting steps and allow excess buffer to collect at the bottom of the tubes after removing most of the supernatant in the first pipetting step.

9. Remove the tubes from the magnet.
10. Add 100 µL of DNA Resuspension Buffer (DR1) to completely resuspend the beads.
11. Add 55 µL (0.55X vol) of Agencourt beads to the 100 µL of resuspended beads.

    Important: Accurate pipetting of bead volume is critical. Avoid carrying excess bead volume from outside of the pipet tip to the sample.

12. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume.
13. Incubate at room temperature for 10 minutes.
14. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
15. Carefully transfer the supernatant to a new tube, taking care not to disturb the beads. After the supernatant is collected, the beads may be discarded.
16. Add 15 µL of Agencourt beads to the 155 µL of supernatant in the new tube. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume.
17. Incubate at room temperature for 10 minutes.
18. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
19. Carefully remove and discard the supernatant, taking care not to disturb the beads.

    Important: It is critical to remove as much of the supernatant as possible. Use at least two pipetting steps and allow excess buffer to collect at the bottom of the tubes after removing most of the supernatant in the first pipetting step.

20. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
21. Remove the 70% ethanol wash using a pipette.
22. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Important: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

23. Air dry the beads on the magnet for 3 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.

24. Remove the tubes from the magnet.
25. Add 20 µL room temperature DNA Resuspension Buffer (DR1) to the dried beads. Mix thoroughly to ensure all beads are resuspended.
26. Transfer the tubes to the magnet and let stand for 3 minutes to completely clear the solution of beads.
27. Carefully remove 20 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.
28. Continue immediately to **Section IV. F. Quantitative and Qualitative Assessment of the Library**.

500 bp size selection protocol

1. If using NuQuant:
   a. Remove diluted NuQuant Standard from 4 °C storage. Mix thoroughly by vortexing, spin and place at room temperature. Protect from light.
   b. Remove NuQuant Buffer from –20 °C and thaw at room temperature. Mix thoroughly by vortexing, spin and place on benchtop.
2. Ensure the Agencourt beads have completely reached room temperature before proceeding.
3. Resuspend the beads by vortexing. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.
4. Add 60 µL (0.6X vol) of Agencourt beads to 100 µL of library product from section **IV. D.**

   Important: Accurate pipetting of bead volume is critical. Avoid carrying excess bead volume from outside of the pipet tip to the sample.
5. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the sample volume.†
6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove and discard the supernatant, taking care not to disturb the beads.

   Important: It is critical to remove as much of the supernatant as possible. Use at least two pipetting steps and allow excess buffer to collect at the bottom of the tubes after removing most of the supernatant in the first pipetting step.
9. Remove the tubes from the magnet.
10. Add 100 µL of DNA Resuspension Buffer (DR1) to completely resuspend the beads.
11. Add 45 µL (0.45X vol) of Agencourt beads to the 100 µL of resuspended beads.

    Important: Accurate pipetting of bead volume is critical. Avoid carrying excess bead volume from outside of the pipet tip to the sample.

† If tubes have become noticeably warped or plastic integrity compromised, transfer entire sample to fresh tubes.

12. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume.
13. Incubate at room temperature for 10 minutes.
14. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
15. Carefully transfer the supernatant to a new tube, taking care not to disturb the beads. After the supernatant is collected, the beads may be discarded.
16. Add 15 µL of Agencourt beads to the 145 µL of supernatant in the new tube. Mix thoroughly by pipetting up and down at least 10 times with a pipettor set to 70% of the Master Mix volume.

    Important: Accurate pipetting of bead volume is critical. Avoid carrying excess bead volume from outside of the pipet tip to the sample.

17. Incubate at room temperature for 10 minutes.
18. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
19. Carefully remove and discard the supernatant, taking care not to disturb the beads.

    Important: It is critical to remove as much of the supernatant as possible. Use at least two pipetting steps and allow excess buffer to collect at the bottom of the tubes after removing most of the supernatant in the first pipetting step.

20. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
21. Remove the 70% ethanol wash using a pipette.
22. Repeat the 70% ethanol wash one more time, for a total of two washes.

    Important: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

23. Air dry the beads on the magnet for 3 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
24. Remove the tubes from the magnet.
25. Add 20 µL room temperature DNA Resuspension Buffer (DR1) to the dried beads. Mix thoroughly to ensure all beads are resuspended.
26. Transfer the tubes to the magnet and let stand for 3 minutes to completely clear the solution of beads.
27. Carefully remove 20 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh set of PCR tubes and place on ice.
28. Continue immediately to Section **IV. F.** Quantitative and Qualitative Assessment of the Library.

D. Frequently Asked Questions (FAQs)

Getting Started

Q1. What materials are provided with Celero EZ DNA-Seq?
Celero EZ DNA-Seq includes all necessary buffers, primers and enzymes for library construction. SPRI purification beads and EvaGreen are not included.

Q2. What equipment is required or will be useful?
A comprehensive list of required and recommended equipment can be found in Section II. B. of the User Guide.

Q3. Can this system be used with other library preparation workflows?
Celero EZ DNA-Seq is an end-to-end solution designed to generate libraries for Illumina sequencing starting from gDNA or cDNA and has not been tested with alternative library preparation systems.

Input Recommendations

Q4. What methods do you recommend for DNA isolation?
We recommend a column-based extraction method, including: Qiagen QIAprep Miniprep or DNeasy Miniprep kits, Zymo Quick-DNA kits and Thermo Fisher PureLink Genomic DNA kits.

Q5. Can I use phenol-chloroform based extractions for DNA isolation?
We do not recommend the use of these methods as any carryover of organics may inhibit downstream enzyme activity. If using, we recommend using a column-based purification of the DNA prior to input into the kit.

Q6. Can I use Celero EZ DNA-Seq with DNA from any organism?
Celero EZ DNA-Seq has been designed for use with a broad range of different organisms. Special consideration should be given when using low-input samples from organisms with large genomes.

Q7. Do I need to use high-quality DNA?
This kit is designed for use with DNA samples of high molecular weight with little or no evidence of degradation. We strongly recommend using high quality DNA with a A260:A280 ratio in excess of 1.8 and A260:A230 ratio in excess of 2.0. Use of DNA samples with lower ratios may result in low library yield.

General Workflow

Q8. How much extra reagent is recommended when preparing the enzymatic fragmentation and adaptor ligation master mixes?
A minimum amount of overage should be used in master mixes to ensure the full nominal number of reactions in the kit. The amount of overage needed depends on sample batch size, pipetting accuracy, and viscosity of reagents. We have found that 12-15% extra volume in the enzymatic fragmentation and adaptor ligation master mixes is sufficient for most experiments.

Q9. Is it necessary to perform enzymatic fragmentation of my DNA?
Yes.

Q10. My input DNA samples are already fragmented (e.g. cfDNA, amplicons). Can I skip the enzymatic fragmentation step?
The enzymatic fragmentation step is required in the Celero EZ-DNA-Seq workflow. For sample types that are already fragmented, Celero DNA-Seq (Part No. 0360) is available with optional mechanical (Covaris) fragmentation and End Repair.

Q11. Can I combine the barcoded libraries prior to the PCR amplification step?
No.

SPRI Bead Purifications

Q12. What is the difference between RNAClean XP and AMPure XP SPRI beads? Can both be used interchangeably?
RNAClean XP beads are certified to be RNase and DNase free. We have tested both RNAClean XP and AMPure XP beads in our kits and observe no difference in performance between products.

Q13. What magnetic separation devices do you recommend for the SPRI bead purifications?
Due to the large number of commercially available magnets, we do not have a comprehensive list of compatible products. However, many magnets are compatible. As long as the magnet is strong enough to clear the solution of magnetic beads, it can be applied to the system. We have the following guidelines for selecting a magnetic separation device:

a. Use a magnet designed for 0.2 mL tubes (PCR tubes), tube strips, or plates. Compared to magnets that are designed for 1.5 mL tubes, these minimize loss that can occur when samples are transferred from one tube to another.

b. Prior to purchasing, check the manufacturer's specifications for minimum and maximum volumes that can be effectively treated.

c. Test the magnet with a mock purification to ensure the magnet will effectively clear the solution under the conditions in the Tecan workflow. This is also helpful to gain familiarity with the purification workflow.

Q14. How can I ensure maximum recovery of sample from the SPRI bead purification?
a. Allow the SPRI beads to reach room temperature before use; cold beads result in lower yields.

b. Ensure that the beads are fully resuspended in solution before adding to the sample.

c. Always use fresh ethanol during the washing steps. When preparing the ethanol, measure out the ethanol and water separately to ensure the desired ethanol concentration is obtained.

d. Mix the bead suspension and sample thoroughly to ensure maximum binding of the samples to the beads.

Library quantification/qualification

Q15. My libraries have been stored at -20 °C. Can I still use NuQuant to quantify my libraries? Can I re-quantify libraries that have been stored at -20 °C?
Yes. Please contact Tecan NGS Technical Support for information on using NuQuant with previously frozen libraries.

Q16. How do I measure my final library yield? Can I use an Agilent Bioanalyzer to evaluate the product?
We recommend using NuQuant to accurately quantify the final libraries for multiplex pooling. The final library pool concentration should be determined using a qPCR-based method before loading onto an Illumina sequencer. Please refer to **section IV. F**. for guidelines on library quantitative and qualitative assessments.

Q17. How many bases do Celero EZ DNA-Seq adaptors add to the library?
The adaptors add 136 bp to the library.

Sequencing Recommendations

Q18. What sequencers are compatible with your libraries?
Celero EZ DNA-Seq libraries are compatible with Illumina sequencing platforms.

Q19. How much material should I load into the sequencer?
Please follow Illumina's recommendations for library QC, quantitation, balancing and loading of the amplified library on the sequencer.

Q20. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?
Each barcode is a minimum edit distance of 3 from any other barcode. This means that a minimum of three edits (replacement, insertion, or deletion) must occur before one barcode becomes a different barcode. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012), Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. *PLoS ONE* **7**(8): e42543. doi:10.1371/ journal.pone.0042543.

Q21. What kind of sequencing primers can I use with your libraries?
Celero EZ DNA-Seq libraries are designed for use with the standard Illumina sequencing primers for both single-end and paired-end sequencing applications.

Q22. Can Celero EZ DNA-Seq libraries be used with paired-end sequencing?
Yes. The libraries produced using this kit can be used for both single-end and paired-end sequencing. Special consideration should be given to the expected insert size in the paired-end assay. The workflow generates libraries with an average insert size that is dependent on the fragmentation conditions used.

Data Analysis

Q23. Are any special considerations needed for how I process Celero EZ DNA-Seq libraries?
The final Celero libraries can be analyzed using standard pipelines. To remove adaptor sequences use standard TruSeq sequences for read trimming.

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