DNA-ClickSeq™ Library Prep

User Guide: For Illumina Sequencing v1.1

ClickSeq
TECHNOLOGIES

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LITERATURE CITATION

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Reference www.clickseqtechnologies.com for the most up-to-date version of this protocol.

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1. Overview

ClickSeq is a simple method for the synthesis of Next-Generation Sequencing (NGS) libraries. ClickSeq derives its names by using 'Click-Chemistry' in the place of common ligation enzymes to 'click-ligate' nucleic acids to sequencing adaptors – an essential and often problematic step in the synthesis of Next-Generation Sequencing cDNA libraries. The process takes advantage of the chain-terminating properties of 3'-azido-nucleotides, which are included the initial *in vitro* reverse-transcription reaction. Commercial reverse transcriptases can also utilize DNA as a template, in addition to RNA. The modified nucleotides are stochastically incorporated into the nascent cDNA, yielding cDNA fragments blocked at their 3' ends with azido groups. The 3'-azido-blocked cDNA fragments are 'click-ligated' onto alkyne-functionalized sequencing adaptors, which can subsequently be PCR-amplified to yield a sequencing-ready NGS library.

ClickSeq is a highly flexible and modular platform for NGS library synthesis where both RNA and DNA templates can be used as input material. ClickSeq offers many advantages over standard DNA NGS protocols as click-chemistry is utilized to attach on the required sequencing adapter, rather than commonly used enzymatic reactions. Overall, this results in increased efficiency of the protocol, fewer processing steps, and reduced time from DNA or RNA to sequencing-ready libraries. Additionally, since there are no fragmentation steps as typically required in common DNA-seq or RNA-seq approaches and because *in vitro* template switching during RT is limited by the chain terminating azido-nucleotides, ClickSeq offers the benefit of ultra-low artifactual chimera rate, with only 3 chimeric events per million reads.[1]

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[1] Routh A, Head SR, Ordoukhanian P, Johnson JE. ClickSeq: Fragmentation-Free Next-Generation Sequencing via Click Ligation of Adaptors to Stochastically Terminated 3'-Azido cDNAs. J Mol Biol. 2015 Aug 14;427(16):2610-6. doi: 10.1016/j.jmb.2015.06.011. Epub 2015 Jun 24. PMID: 26116762; PMCID: PMC4523409.

2. Schematic Overview

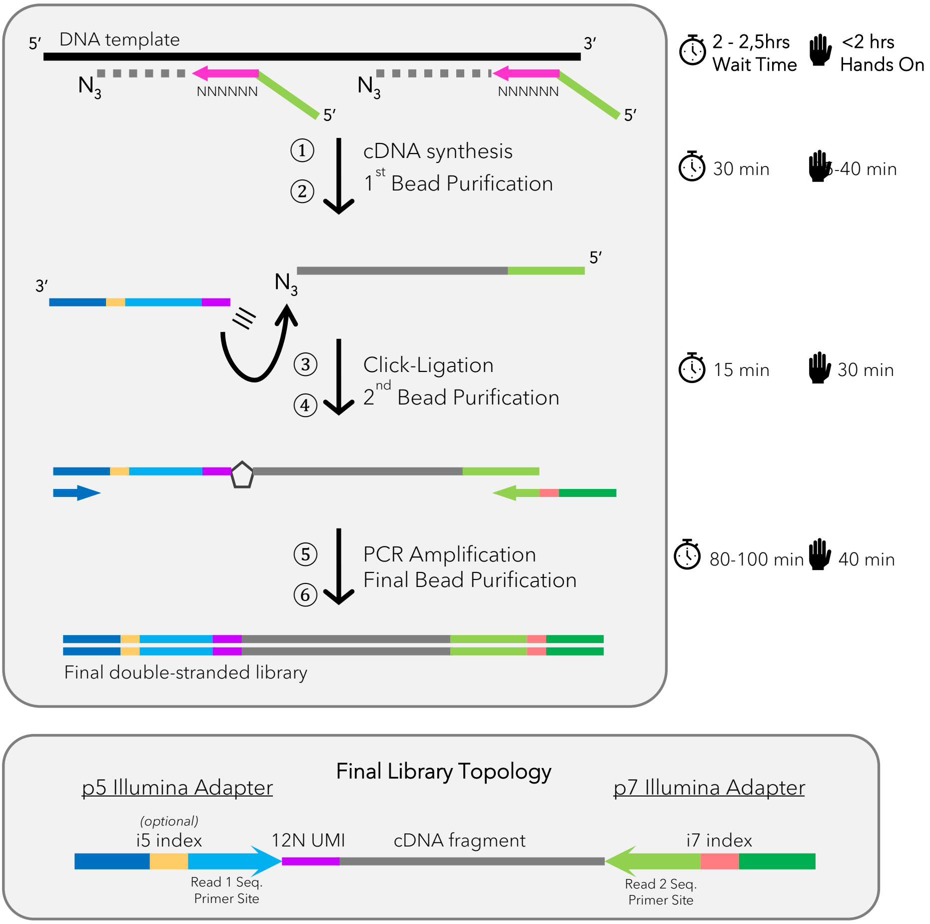
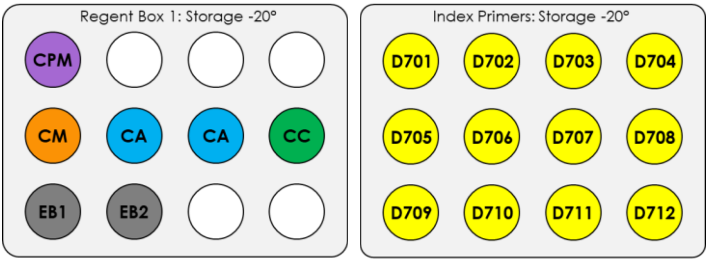


Figure 1: Flowchart of the ClickSeq™ method for library preparation. ① cDNA is synthesized from DNA using reverse transcriptase and a 6N-primer that contains a partial sequence of the Illumina p7 sequencing adapter. cDNA synthesis is completed in the presence of azido-nucleotides that stochastically terminate cDNA chain extension. ② cDNA is purified using SPRI magnetic beads. ③ Click-chemistry is then utilized to chemically ligate the Illumina p5 sequencing adapter onto the cDNA fragment. ④ Click-ligated cDNA is then purified using magnetic SPRI beads. ⑤ PCR amplification is used to attach the remainder of the p7 adapter (that contains the sample index) and to generate sufficient material for loading onto an Illumina flowcell. ⑥ A final bead purification and size selection is done resulting in sequencing ready libraries. The final library topology includes both Illumina p5 and p7 sequencing adapters, single or dual index sequences, a 4nt UMI (unique molecular identifier), and the cDNA fragment. The first read during sequencing reads through the UMI and across the cDNA fragment.

3. Kit Components



Component	Label	Volume (µl) *(12 reactions)*	Storage
Click Primer Mix	CPM 🟪	40	-20°
Click Mix	CM 🟧	230	-20°
Click Accelerant	CA 🟦	400 (x2)	-20°
Click Catalyst	CC 🟩	60	-20°
i7 Index Primers (D7xx-D7xx)	D7xx 🟨	35	-20°
Elution Buffer 1	EB1 ⬜	600	-20°/+4°
Elution Buffer 2	EB2 ⬜	1200	-20°/+4°

- ➢ Upon receival of the ClickSeq kit store Reagent Box 1 and Index Primers Box should be stored at -20°C.
- ➢ Note: As per manufacture requirements, enzymes (i.e. SSIII, One*Taq* Polymerase) should be thawed and kept on ice while in use. All other reagents can be thawed at room temperature. Index Primers are not included in the kit and can be purchased separately (BCK-RNAseq-IP)
- ➢ Symbology: ⚠ Indicates a step where extra caution should be taken; 🛑 Indicates a good stopping point within the protocol, samples can be stored at -20° and the protocol resumed at a future time.

4. User-Supplied Equipment and Consumables

Check to ensure that all the necessary components, materials, and equipment are present before beginning this protocol.

Reagents

- SuperScript III™ Reverse Transcriptase, 200U/μL (Invitrogen; 18080-093 or 18080-044)
- One*Taq*® 2X Master Mix with Standard Buffer (NEB; M0482S or M0482L) (⚠ Note: you ***must*** use One*Taq* for this step, this enzyme cannot be substituted for a different PCR enzyme)
- SPRIselect (Beckman Coulter; B23317) or equivalent DNA/RNA Purification Beads (also known as SPRI beads)
- Nuclease free water
- 80% ethanol (made fresh)

Equipment

- UV-spectrophotometer or equivalent (for DNA quantification)
- Single-channel pipettes (1 - 1000μl)
- [optional] Multi-channel pipettes (1 - 200μl)
- Thermocycler
- Magnetic rack, for 0.2mL PCR strip tubes or 96-well plates
- Benchtop centrifuge (for quick-spin collection of reagents, 1.5mL tube and 0.2mL PCR strip compatible)
- Vortex
- Ice box or equivalent
- [optional] Qubit fluorometer, with dsDNA High Sensitivity reagents
- [optional] Agilent BioAnalyzer, with High Sensitivity DNA reagents and chip

Labware

- Tips, low-binding (aerosol barrier recommended)
- 1.5mL tubes, low-binding recommended
- 0.2mL thin-walled PCR tubes or 96-well plates with caps or sealing film

5. Guidelines

Next-Generation Sequencing (NGS) is a highly sensitive technique that generates millions of data points. The quality of your input material can be translated to the final quality of your libraries at the end of this protocol, and in turn, the sequencing data. Use common sense laboratory precautions to minimize introducing contamination to your samples and follow procedures as written to ensure good yields.

DNA Handling

- Wear PPE (gloves, lab coat, etc) to protect your workstation and reagents from nucleases that are present on your skin. Change gloves often.

Bead Handling

- Follow manufacturers recommendations but generally, SPRI purification beads should be stored at +4°C. Beads tend to settle during storage so they should be resuspended thoroughly before use (by vortexing or pipetting vigorously). Beads are resuspended properly when the solution is uniform in color (light brown) and there are no visible clumps on the bottom or sides of the tube.
- SPRI beads are magnetic and are collected by placing the sample tube on a magnetic rack. The time it takes for the beads to pellet will depend on the strength of the magnet you are using; adjust the incubation time accordingly by waiting until the solution is completely clear. Waiting longer to ensure that all the beads have pelleted will not affect overall quality of your libraries but will ensure adequate efficiency of the purification steps.
- When discarding the supernatant of pelleted beads, take care to not disturb the beads by keeping the sample tube on the magnetic rack and do not touch the pellet with a pipette tip.
- ⚠ Ethanol carryover after the second wash step during bead purification can inhibit subsequent reactions. Visually inspect each well to ensure all ethanol has been removed.
- Do not allow beads to over-dry, exhibited by visible cracking. This can damage the beads and reduce overall yields.

General

- Read an entire section of the protocol before beginning to familiarize yourself with all steps. To minimize any issues, collect the necessary equipment, prepare the appropriate reagents, and pre-load the appropriate incubation temperatures on your thermocycler.

- Enzymes should be thawed and kept on ice while in use. All other reagents can be thawed at room temperature and kept on ice while not in use. SPRI beads equilibrate to room temperature prior to use.
- Spin down all reagent tubes prior to opening to prevent loss and to minimize cross-contamination.
- Use calibrated pipettes and fresh tips between samples and reagents.
- Pipette reagents and mixes carefully and in a controlled manner. Viscous reagents (such as enzyme mixes) should be pipetted slowly to ensure accuracy and the complete transfer of the reagent. Avoid frothing and the introduction of air bubbles while mixing.

Master Mixes

- Steps #4 and #16 require the generation of master mixes. In order to have enough solution for all samples, include a 10% surplus per reaction when calculating the master mix.

$$volume\ to\ add\ to\ mix\ (\mu l) = per\ rxn\ volume\ (\mu l)\ x\ number\ of\ samples\ x\ 1.1$$

6. Protocol

① cDNA synthesis

DNA is copied by reverse transcriptase by randomly priming using a 6N primer with a partial p7 Illumina adaptor sequence. The presence of AzNTPs stochastically terminates cDNA chain extension generating a distribution of randomly sized cDNA fragments. Note: *The random primers in Step 1 will bind to any nucleic acid present in your sample (RNA or DNA). The material you use as input will be generated into sequencing libraries.*

1. In a 0.2ml tube, dilute 100ng-1µg* of input DNA to 11µl using nuclease free water. *Reference Appendix A for input DNA considerations.
2. Add 3µl of ClickSeq Primer Mix **(CPM 🟪)** to the diluted DNA. Mix well.
3. Incubate the mixture at 95°C for 2 mins to melt DNA and immediately snap cool the reaction by placing the tubes on ice for >1 min to anneal the reverse primer.
4. After snap cooling, generate an RT master mix in a separate tube by combining the following components, pipette well to mix:

	Per Rxn	Master Mix
SSIIRT - 5X First Strand Buffer	4µl	
SSIIRT - DTT (100mM)	1µl	
SSIII Reverse Transcriptase™	1µl	

5. Add 6µl of the RT master mix to each reaction and pipette to mix.
6. Incubate the reaction in a thermocycler using the following conditions:
   25°C for 10 mins, 50°C for 10 mins, 75°C for 10 mins, 4°C for ∞.

➢ Note: At this point it is recommended to remove the SPRI Bead reagents from 4°C storage to allow them to equilibrate to room temperature.

② First Bead Purification

Magnetic beads are used to remove components of the reverse transcription reaction leaving the cDNA fragments for further processing.

- ➢ Note: SPRI beads tend to settle during storage and should be thoroughly resuspended by vortexing briefly prior to use. Additionally, it is important to allow the SPRI beads to equilibrate to room temperature for 30 min.

7. Add 36µl of thoroughly resuspended SPRI beads to the reaction mix. Mix well by pipetting. Incubate for 5 min at room temperature.
8. Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 5 min or until the supernatant is completely clear.
9. Leaving the sample tubes on the magnetic rack, discard clear supernatant taking care to not disturb the pelleted beads.
10. Leaving the sample tubes on the magnetic rack, wash pelleted beads by adding 200µl of freshly prepared 80% EtOH. Do not resuspend beads. After 30 seconds incubation, remove and discard the supernatant.
11. Repeat the EtOH washing step (#10) for a total of two washes. After the second wash make sure to remove all traces of EtOH as ethanol can impair the efficiency of subsequent steps. Visually inspect tubes for trace amounts of EtOH left over on the sides of the tubes. Tubes should be removed off the magnet and pulse spun to collect extra EtOH at the bottom of the sample tube. Place tubes back on a magnetic stand and pipette off any remaining EtOH.
12. Remove the sample tubes from the magnetic rack and resuspend the beads by adding 21µl of Elution Buffer 1 (EB1 □). Incubate resuspended beads for 2 min at room temperature.
13. Place the sample tubes back on the magnetic rack and allow beads to pellet. Transfer 20µl of the supernatant to a fresh 0.2ml sample tube.

- ➢ 🛑 Note: This is a safe stopping point. Samples can be stored at -20°C.

③ Click-Ligation

During this step, a sequencing adapter is attached to the azido-terminated 3′ ends of the cDNA fragments using a Click-Chemistry reaction.

⚠ **It is important to complete the Click-Ligation reaction, steps #15-16, in a timely manner**, this is to avoid oxidation of the Click Accelerant 🟦 by atmospheric oxygen.

- ➢ Note: At this point it is recommended to remove the SPRI bead reagents from 4°C storage to allow them to equilibrate to room temperature.

14. Add 15µl of Click Mix **(CM 🟧)** to each sample. Pipette to mix, taking care to not introduce air bubbles.
15. **In a separate tube**, prepare the Click Ligation master mix, pipetting up and down 3-5 times to mix, taking care to not introduce any air bubbles:

	Per Rxn	Master Mix
Click Accelerant **(CA 🟦)**	4µl	
Click Catalyst **(CC 🟩)**	1µl	

⚠ **CC** is blue in color and should turn clear/colorless when mixed properly. This is a time sensitive reaction so **proceed immediately to the next step**.

- ➢ Note: The tube containing CA 🟦 should only be used one time to limit exposure to atmospheric oxygen. Discard the tube once it has been used. Two tubes have been provided if the Click-Reaction needs to be completed at two separate times.

16. Add 5µl of the Click Ligation master mix to each sample tube. Once the mix has been added to all sample tubes, pipette or flick to mix, and spin down contents of the tube. Incubate the reaction at room temperature for 15 min.

④ Second Bead Purification

SPRI beads are used to remove components of the click-ligation reaction leaving cDNA fragments that are flanked by sequencing adapters.

- ➢ Note: SPRI beads tend to settle during storage and should be thoroughly resuspended by vortexing briefly prior to use. Additionally, it is important to allow beads to equilibrate to room temperature for 30 min.

17. Add 64µl of thoroughly resuspended SPRI beads to the reaction mix. Mix well by pipetting. Incubate for 5 min at room temperature.
18. Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 5 min or until the supernatant is completely clear.
19. Leaving the sample tubes on the magnetic rack, discard clear supernatant. Take care to not disturb the pelleted beads.
20. Leaving the sample tubes on the magnetic rack, wash pelleted beads by adding 200µl of freshly prepared 80% EtOH. Do not resuspend beads. After 30 seconds incubation remove and discard the supernatant.
21. Repeat the EtOH wash step (#20) for a total of two washes. After the second wash make sure to remove all traces of EtOH as ethanol can impair the efficiency of subsequent steps. Visually inspect tubes for trace amounts of EtOH left over on the sides of the tubes. Tubes should be removed off the magnet and pulse spun to collect extra EtOH at the bottom of the tube. Place tubes back on a magnetic stand and pipette off any remaining EtOH.
22. Remove the sample tubes off the magnetic rack and resuspend the beads by adding 21µl of Elution Buffer 2 (EB2 ▢). Incubate resuspended beads for 2 min at room temperature.
23. Place the sample tubes back on the magnetic plate and allow beads to pellet. Transfer 20µl of the supernatant to a fresh 0.2ml PCR tube.

- ➢ 🛑 Note: This is a safe stopping point. Samples can be stored at -20°C.

⑤ PCR Library Amplification

At this step, PCR is used to convert the single-stranded cDNA fragments to dsDNA fragments, amplify the fragments to generate enough material for sequencing, and to add the sequencing indices/barcodes (Illumina i7 adapters).

24. Transfer 10µl of the sample volume to a new PCR tube. (Note: Retain the other 10µl of your sample. It can be used to repeat the PCR amplification step in the case of over- or under-cycling or for technical replicates).
25. Add 25µl of One*Taq*® 2X Master Mix to each sample tube.
26. Using a unique Index Primer per sample, add 15µl of each respective i7 index primer (Index Primer D701-D712 🟨) to each sample tube. Pipette to mix. Take note of which index was used for each sample.
27. Place the sample tubes in a thermocycler using the following PCR cycling program:
    - 94°C 1min; 53°C 30sec; 68°C 10min;
    - [94°C 30sec, 53°C 30sec, 68°C 2min] x 12-21 cycles*;
    - 68°C 5min; 4° ∞

*Reference Appendix B to determine the appropriate cycle number for your application. 18 cycles is recommended for most applications or samples.

- 🛑 Note: This is a safe stopping point. Samples can be left in the thermocycler at 4°C overnight, or stored at -20°C. If you choose to proceed with the protocol, at this point it is recommended to remove the SPRI bead reagents from 4°C storage to allow them to equilibrate to room temperature.
- In the case of over- or under-cycling, starting at step #24, the protocol may be repeated using the retained 10µl of your sample. Under-cycling will result in low yield and the PCR amplification should be repeated with a higher cycle number. Over-cycling can result in excessive PCR duplication and will result in excess library.

⑥ Final Bead Purification

Magnetic bead purification is used to remove components of the PCR amplification reaction from the completed barcoded libraries and return size-selected (~300-600bp) sequencing-ready libraries. If more specific size-selection is required, please reference Appendix C for an additional size selection step.

- ➢ Note: SPRI beads tend to settle during storage and should be thoroughly resuspended by vortexing briefly prior to use. Additionally, it is important to allow SPRI beads to equilibrize to room temperature for ~30 min.

28. Add 30µl of thoroughly resuspended SPRI beads to the reaction mix. Mix well by pipetting. Incubate for 5 min at room temperature.
29. Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 5 min or until the supernatant is completely clear.
30. ⚠ Leaving the sample tubes on the magnetic rack, **transfer the supernatant to fresh 0.2ml tubes.** Take care to not disturb the pelleted beads. Pelleted beads may be discarded. Do NOT discard the supernatant.
31. Add 15µl of thoroughly resuspended SPRI beads to the retained supernatant from step #31. Mix well by pipetting. Incubate for 5 min at room temperature.
32. Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 5 min or until the supernatant is completely clear.
33. Leaving the sample tubes on the magnetic rack, discard clear supernatant. Take care to not disturb the pelleted beads.
34. Leaving the sample tubes on the magnetic rack, wash pelleted beads by adding 200µl of freshly prepared 80% EtOH. Do not resuspend beads. After 30 seconds incubation, remove and discard the supernatant.
35. Repeat the EtOH wash step (#35) for a total of two washes. After the second wash, make sure to remove all traces of EtOH as ethanol can impair the efficiency of subsequent steps. Visually inspect tubes for trace amounts of EtOH left over on the sides of the tubes. Tubes should be removed off the magnet and pulse spun to collect extra EtOH at the bottom of the tube. Place tubes back on a magnetic stand and pipette off any remaining EtOH.
36. Remove the sample tubes off the magnetic rack and resuspend the beads by adding 18µl of Elution Buffer 2 **(EB2 □)**. Incubate resuspended beads for 2 min at room temperature.
37. Place the sample tubes back on the magnetic plate and allow beads to pellet. Transfer 17µl of the supernatant to a fresh tube.
38. Samples are now ready for quality control, quantification, pooling, and sequencing. Reference Appendix D for sequencing guidelines.

- ➢ 🛑 Note: This is a safe stopping point. Samples can be stored at -20°C.

7. Short Procedure

① cDNA Synthesis

☐ Dilute 0.1-1µg of DNA to 11µL using H_2O

☐ Add 3µL CPM 🟪 to each sample

☐ Incubate 95°C for 2 mins, then snap cool by placing tubes on ice

☐ Prepare a RT master mix of 4µL 5X buffer, 1µL DTT, and 1µL SSIII

☐ Add 6µL of the RT Master Mix to each sample

☐ Incubate: 25°C for 10 mins, 50°C for 10 mins, 75°C for 10 mins, 4°C for ∞

② First Bead Purification

☐ Add 36µL SPRI Beads to each sample

☐ Incubate at room temperature for ~5 min

☐ Place sample tubes on a magnetic rack and pellet beads, ~2-5min

☐ Discard supernatant, do not disturb beads

☐ Wash pelleted beads twice with 200µL 80% EtOH

☐ Remove beads from magnetic stand and resuspend beads in 21µL EB1 ⬜

☐ Incubate at room temperature for 2 min

☐ Place samples on a magnetic rack, allow beads to pellet

☐ transfer 20µL of the supernatant to a fresh sample tube [safe stopping point]

③ Click Ligation

☐ Add 15µL CM 🟧 to each sample

☐ Prepare a master mix of 4µL CA 🟦 and 1µL CC 🟩. Proceed to next step immediately.

☐ Add 5µL of the CA/CC mix to each sample

☐ Incubate at room temperature for 15min

④ Second Bead Purification

☐ Add 64µL SPRI Beads to each sample

☐ Incubate at room temperature for ~5 min

☐ Place sample tubes on a magnetic rack and pellet beads, ~2-5min

☐ Discard supernatant, do not disturb beads

☐ Wash pelleted beads twice with 200µL 80% EtOH

☐ Remove beads from magnetic stand and resuspend beads in 21µL EB2 ⬜

☐ Incubate at room temperature for 2 min

☐ Place samples on magnetic rack, allow beads to pellet

☐ transfer 20µL of the supernatant to a fresh sample tube [safe stopping point]

Continued on next page.

⑤ PCR AMPLIFICATION

☐ Transfer 10µL of the sample to a new sample tube

☐ Add 25µL OneTaq 2X Master Mix to each sample

☐ Using a unique index per sample, add 15µL of Index Primer **D701-D712** 🟨 to each sample

☐ Using a thermocycler, incubate samples using the following protocol:
94°C 1min; 53°C 30sec; 68°C 10min;
[94°C 30sec, 53°C 30sec, 68°C 2min] x12-21cycles;
68°C 5min; 4° ∞
[safe stopping point; samples can be left in thermocycler overnight or at -20°]

⑥ FINAL BEAD PURIFICATION

☐ Add 30µL SPRI Beads to each sample

☐ Incubate at room temperature for ~5 min

☐ Place sample tubes on a magnetic rack and pellet beads, ~2-5min

☐ Retain supernatant and transfer to new PCR tubes, do not disturb beads

☐ Add 15µL SPRI Beads to retained supernatant

☐ Incubate at room temperature for ~5 min

☐ Place sample tubes on a magnetic rack and pellet beads, ~2-5min

☐ Discard supernatant, do not disturb beads

☐ Wash pelleted beads twice with 200µL 80% EtOH

☐ Remove beads from magnetic stand and resuspend beads in 18µL **EB2** ⬜

☐ Incubate at room temperature for 2 min

☐ Place samples on magnetic rack, allow beads to pellet

☐ Transfer 17µL of the supernatant to a fresh sample tube

☐ Samples are now ready for QC, quantification, pooling, and sequencing
[safe stopping point]

8. Appendix A: DNA Input Guidelines

DNA Input, Quantification, and Quality Control Guidelines

- Most standard DNA extraction protocols are compatible with this method. Take care during the final steps of the extraction method to ensure that no salts, metal ions, or organic solvents are carried over into the final elution step. ⚠ For example, ethanol contamination can reduce the efficiency of all reactions in this protocol.
- During the DNA extraction elute your sample in nuclease-free water or Tris buffer (10mM, pH 7.4). ⚠ We recommend you do NOT use carrier DNA during the extraction process. These will negatively impact your final sequencing data as the carrier DNA will also be sequenced.
- When using ClickSeq for DNAseq applications, we recommend using protocols that do not co-purify RNA, since this may also provide a substrate for the RT reaction.
- The random primer of this ClickSeq kit will generate cDNA fragments off any nucleic acid in your sample. ⚠ WHAT YOU PUT IN THE INTIAL REACTION (Step #1) IS WHAT YOU WILL SEQUENCE. Make sure to purify your samples prior to starting this protocol
- DNA can be quantified by any of the user's preferred methods (UV-vis spectrophotometer, Qubit fluorometer, etc.)
- This protocol has been demonstrated to work with as little as 10ng up to 4µg of DNA. That quantity should be in a max of 11µl water or Tris-buffer. While it is possible to use the specified range of starting material, we have found that the optimal amount to start with is generally >100ng of DNA. Below this value, additional PCR cycles are required that will result in PCR duplication and increased adaptor-dimers in the final library.
- A260/A280 values should be between 1.6 and 1.8

9. Appendix B: PCR Cycle Number

Step ⑤ of this protocol includes PCR amplification. This step not only adds on the remainder of the Illumina sequencing adapter and index barcode to each sample, but it also amplifies your fragments to ensure that you have enough material to load onto the sequencing platform (Illumina) flowcell.

Optimizing the number of PCR cycles for your sample type increases the overall quality of the final sequencing data. Over-cycling can result in an increase in PCR duplicates leading to less accurate read counting, while under-cycling can result in insufficient library yield. Duplication can be bioinformatically addressed using the UMIs built into the Click-Adaptor but will result in the reduction of usable read counts.

Final Library Target Quantification

The final library target quantification will depend on the Illumina sequencing platform. Please refer to the respective platform's '*sample loading guidelines*' or contact your sequencing core specialist to determine what your target quantification should be.

- ➢ For example: The NextSeq550 is a common sequencing platform used in many sequencing/genomics cores. For this platform, Illumina recommends a starting library concentration of 0.5nM – 4nM.
- o For this application we recommend aiming for a target of 3nM (or ~0.6ng/µl for an average 300bp fragment size).
- o Molarity is calculated using the following equation:

$$nM = \frac{library\ concentration\ \left(\frac{ng}{\mu l}\right) x\ \ 10^6}{average\ library\ size\ (bp)\ x\ 660}$$

- ➢ Individual samples should be quantified using a BioAnalyzer, with a Qubit Fluorometer, or with qPCR. The final library pool should be quantified using qPCR using primers that bind to the Illumina sequencing adapters to ensure the most accurate loading quantification.

PCR Cycle Optimization

The number of PCR cycles to perform will depend on the sample type (species, tissue, quality, RNA vs DNA, etc.) so optimizations should be completed prior to processing all samples of the same type. This protocol has been extensively tested using total cellular RNA extracted from *D. melanogaster* (S2) cells. The provided values should be used as a reference only.

TOTAL DNA INPUT	PCR CYCLE NUMBER
20ng	18-21
50ng	17-20
100ng	16-19
250ng	12-18

10. Appendix C: Library Size Selection

Step ⑥ of this protocol includes a size selection step using SPRI beads. Following the protocol as directed will retain fragments ~300-600bp. This is the optimal size fragment size range for the ClickSeq protocol. The final fragments consist of: ~140nt of sequencing adapters and ~160-460nt of the cDNA fragment. If a tighter size selection is required, we suggest following the protocol below.

Equipment and Consumables

- Agarose gel electrophoresis system and corresponding reagents (gel bed cassettes, gel combs, tank, power supply, agarose, running buffers, loading dyes, DNA stain (DNA safe stains are recommended such as SYBR), etc)
- dsDNA Ladder with 400 and 600bp resolution marks
- UV-transilluminator or equivalent depending on DNA stain used (blue light transilluminator is recommended)
- Clean scalpel or razor blade or equivalent; for excising samples run on agarose gel
- 1.5mL tubes, low-bind recommended
- User defined agarose dissolving and DNA extraction protocol, and corresponding equipment and reagents (e.g. Zymo DNA Extraction Kit)

Agarose Gel DNA Extraction Protocol

Complete steps **#1-38** of this protocol as directed then follow the modified protocol:

40. Quantify samples using the Qubit dsDNA High Sensitivity Kit or with a BioAnalyzer High Sensitivity DNA kit.
41. Make an equimolar pool(s) of your samples (or however you would like to distribute the pool). You may pool all your samples into one pool, or you may make a few pools with fewer samples per pool. When pooling, consider the capacity of your gel electrophoresis system.
42. Following the protocol for the agarose system of your choosing; assemble your gel electrophoresis system, mix your sample with loading dye (if necessary), load your samples and ladder into the wells of your gel, and run the gel to separate your samples.
43. Using a clean gel knife and referencing the DNA ladder, excise the gel between 300 and 600bp (or as required per the user's assay).
44. Following the user defined agarose DNA extraction protocol, dissolve and extract the DNA from the excised agarose. Elute/resuspend your sample in 10-20µl of Elution Buffer 2 (**EB2** ⬜).
45. Samples are now ready for quality control, quantification, pooling, and sequencing. Reference Appendix D for sequencing guidelines.

11. Appendix D: Sequencing Guidelines

Final ClickSeq libraries are compatible with the Illumina sequencing platforms (NextSeq, NovaSeq, MiSeq, HiSeq, etc.) and do not require any custom sequencing primers.

- It is not recommended to multiplex ClickSeq libraries with other libraries made with other techniques or from other vendors. While the sequencing adapters are compatible among the different techniques, the final library size and topology can have unwanted effects on the quality and output of the final read data.
- Read 1 will include 4nt of the UMI followed by the cDNA fragment.
- The 8nt i7 index sequences are provided in the table below.

INDEX	SEQUENCE
D701	ATTACTCG
D702	TCCGGAGA
D703	CGCTCATT
D704	GAGATTCC
D705	ATTCAGAA
D706	GAATTCGT
D707	CTGAAGCT
D708	TAATGCGC
D709	CGGCTATG
D710	TCCGCGAA
D711	TCTCGCGC
D712	AGCGATAG

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