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ClickSeq: Random-Primed Protocol with Single Indexing using ClickSeq Kit

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External link: **https://www.clickseqtechnologies.com/kits**

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Disclaimer

ClickSeq™ is a pending trademark of ClickSeq Technologies, LLC.

Abstract

ClickSeq is a simple, fragmentation-free method for the synthesis of Next-Generation Sequencing (NGS) libraries. ClickSeq derives its name 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 reactions uniformly required for RNAseq. 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 RNA 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 RNA to sequencing ready libraries. Additionally, since there are no fragmentation steps typically required in common 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.

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.

RNA Handling

- Work in an RNase-free environment; use RNase inhibitors to decontaminate your workspace. Follow standard aseptic techniques.
- Wear PPE (gloves, lab coat, etc) to protect your workstation and reagents from RNases that are present on your skin. Change gloves often.
- Use RNase-free plasticware by purchasing certified materials or by treating consumables with RNase Inhibitors.

RNA Input Guidelines

- Most standard RNA 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 RNA extraction elute your sample in RNase-free water or Tris buffer (10mM, pH 7.4). We recommend you do NOT use carrier RNA during the extraction process. These will negatively impact your final sequencing data as the carrier RNA will also be sequenced.
- When using ClickSeq for RNAseq applications, we recommend using protocols that do not co-purify genomic DNA, since this may also provide a substrate for the RT reaction. If available, complete the specified DNase I treatment during RNA extraction.
- 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 (i.e. rRNA deplete, polyA-select, or another purification method that suits your needs).
- RNA 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 RNA. That quantity should be in a max of 10µl 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 RNA. 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 1.9 and 2.2
- RIN values should be >6.0

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 #17 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.

PCR Cycle Optimization

The number of PCR cycles to perform will depend on the sample type (species, tissue, quality, 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 RNA Input	PCR Cycle Number
20ng	18-21
50ng	17-20
100ng	16-19
250ng	12-18

Sequencing Guidelines

- Final ClickSeq libraries are compatible with the Illumina sequencing platforms (NextSeq, NovaSeq, MiSeq, HiSeq, etc.) or with Element Biosciences Aviti™ systems/flowcells that are compatible with the same Illumina adaptors.
- Final ClickSeq libraries can also be sequenced on Oxford Nanopore Technology's flowcell by straight-forward ligation of the ONT DNA sequencing (e.g. LSK109) adaptors.
- Read 1 will include 4nt of the UMI followed by the cDNA fragment.
- The 8nt i7 index sequences are provided in the table below.

	A	B
	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

Materials

Required Reagents

- ClickSeq Kit **ClickSeq Technologies** (https://www.clickseqtechnologies.com/kits)
- SuperScript™ III Reverse Transcriptase **Thermo Fisher Catalog #**18080093
- OneTaq 2X Master Mix with Standard Buffer - 100 rxns **New England Biolabs Catalog #**M0482S
- SPRIselect reagent kit **Beckman Coulter Catalog #**B23317 or equivalent DNA/RNA Purification Beads
- Nuclease-free water
- 80% ethanol (made fresh)

Optional Reagents

- RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #**10777019
- RNase H - 250 units **New England Biolabs Catalog #**M0297S

Protocol materials

SuperScript™ III Reverse Transcriptase **Thermo Fisher Catalog #**18080093	Materials
RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #**10777019	Materials
RNase H - 250 units **New England Biolabs Catalog #**M0297S	Materials, Step 7
ClickSeq Kit **ClickSeq Technologies LLC**	Materials
OneTaq 2X Master Mix with Standard Buffer - 100 rxns **New England Biolabs Catalog #**M0482S	Materials, Step 26
SPRIselect reagent kit **Beckman Coulter Catalog #**B23317	In Materials and 4 steps

Safety warnings

Standard molecular lab precautions should be adhered to, including standard PPE (gloves, lab coat, eye protection, etc).

Before start

Check to ensure that you have all the necessary components, materials, and equipment before beginning this protocol. The protocol can be grouped into 6 broad steps/sections:

1. **Reverse Transcription and RNA Removal:** RNA is reverse transcribed 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.

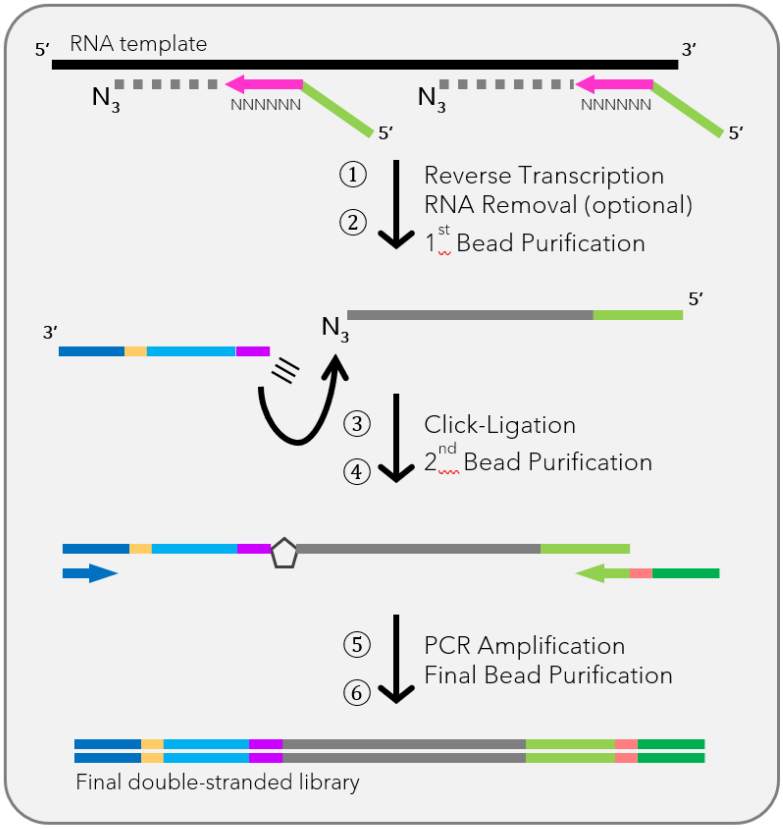
2. **First Bead Purification:** Magnetic beads are used to remove all components of the reverse transcription reaction leaving the cDNA fragments for further processing.

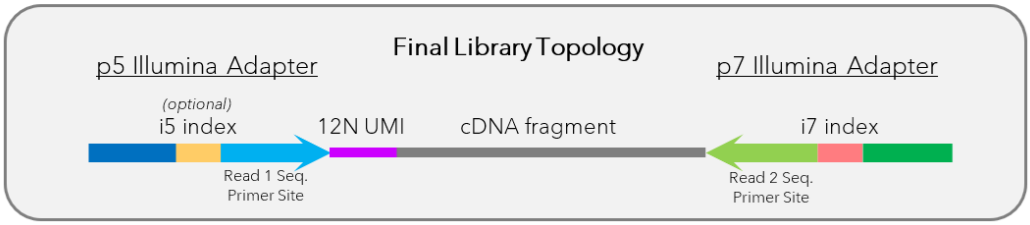
3. **Click-Ligation:** During this step, a sequencing adapter is attached to the azido-terminated 3’ ends of the cDNA fragments using a Click-Chemistry reaction.

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.

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).

6. **Final Bead Purification:** Magnetic bead purification is used to remove all components of the PCR amplification reaction from the completed barcoded libraries.





ClickSeq Schematic

Reverse Transcription and RNA Removal

50m

1 In a 0.2ml tube, dilute 100 ng - 1 µg of input RNA to 10 µL using nuclease free water.

Note

This protocol has been demonstrated to work with as little as 10ng up to 4µg of RNA. That quantity should be in a max of 10µl 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 RNA. Below this value, additional PCR cycles are required that will result in PCR duplication and increased adaptor-dimers in the final library.

The random primers in Step 2 will bind to any nucleic acid present in your sample. All the material you use as input will be included into the final sequencing libraries. Use poly(A) selected, rRNA depleted samples, or another type of purification method for your input RNA.

2 Add 3 µL of ClickSeq Primer Mix (CPM) to the diluted RNA. Mix well.

Note

The primer used in this step is:
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNN

3 Incubate the mixture at 65 °C for 00:05:00 to melt any RNA secondary structure and immediately snap cool the reaction by placing the tubes on ice for 00:01:00 to anneal the reverse primer.

6m

4 After snap cooling, generate an RT master mix in a separate tube by combining the following components, pipette well to mix:

	A	B	C
		Per Rxn	**Master Mix**
	5X First Strand Buffer	4µl	
	DTT (100mM)	1µl	
	[optional] Recombinant Ribonuclease	1µl	

	A	B	C
	Inhibitor [or nuclea se-free water]		
	SSIII Reverse Trans criptase	1µl	

Generate a Master Mix of these components when preparing more than one sample at a time

Note

Recombinant Ribonuclease Inhibitor is not essential in cases where RNA quality or abundance is not a concern and can be replaced with nuclease-free water to save on reagent costs

5 Add 7 µL of the RT master mix to each reaction and pipette to mix.

6 Incubate the reaction in a thermocycler using the following conditions: 30m

25 °C for 00:10:00
50 °C for 00:10:00
75 °C for 00:10:00
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.

7 [optional] To remove template RNA, add 0.5 µL of 30m

RNase H - 250 units **New England Biolabs Catalog #**M0297S . Pipette to mix.

Incubate the reaction in a thermocycler using the following conditions:

37 °C for 00:20:00
80 °C for 00:10:00
4 °C for ∞

Note

Removal of the RNA template using RNaseH is not essential but may improve library yield in some cases. In cases where library yield is not a concern, step #7 may be omitted to save on reagent costs and prep time.

First Bead Purification

15m

8 Add 36 µL of thoroughly resuspended SPRIselect reagent kit **Beckman Coulter Catalog #**B23317 beads to the reaction mix. Mix well by pipetting. Incubate for 00:05:00 at Room temperature .

5m

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.

9 Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 00:05:00 or until the supernatant is completely clear.

5m

10 Leaving the sample tubes on the magnetic rack, discard clear supernatant taking care to not disturb the pelleted beads.

11 Leaving the sample tubes on the magnetic rack, wash pelleted beads by adding 200 µL of freshly prepared 80% EtOH. Do not resuspend beads. After 00:00:30 incubation remove and discard the supernatant.

30s

12 Repeat the EtOH washing step (#11) 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.

13 Remove the sample tubes from the magnetic rack and resuspend the beads by adding 21 µL of Elution Buffer 1 **(EB1)**. Incubate resuspended beads for 00:02:00 at Room temperature

2m

14 Place the sample tubes back on the magnetic rack and allow beads to pellet. Transfer 20 µL of the supernatant to a fresh 0.2ml sample tube.

Safe stopping point: samples can be stored at -20 °C

Click-Ligation

20m

15 Add 15 µL of Click Mix **(CM)** to each sample. Pipette to mix, taking care to not introduce air bubbles.

Note

The Click-Adaptor present in the Click Mix **(CM)** is:
5' Hexynyl-NNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT

16 **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. **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**.

	A	B	C
		Per Rxn	**Master Mix**
	Click Accelerant **(CA)**	4µl	
	Click Catalyst **(CC)**	1µl	

Generate a Master Mix of these components when preparing more than one sample at a time

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. The ClickSeq kit provides two CA tubes.

17 Add 5 µL 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

15m

the reaction at room temperature for 00:15:00 .

Second Bead Purification

15m

18 Add 64 µL of thoroughly resuspended SPRIselect reagent kit **Beckman Coulter Catalog #**B23317 beads to the reaction mix. Mix well by pipetting. Incubate for 00:05:00 at Room temperature

5m

19 Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 00:05:00 or until the supernatant is completely clear.

5m

20 Leaving the sample tubes on the magnetic rack, discard clear supernatant. Take care to not disturb the pelleted beads.

21 Leaving the sample tubes on the magnetic rack, wash pelleted beads by adding 200 µL of freshly prepared 80% EtOH. Do not resuspend beads. After 00:00:30 incubation remove and discard the supernatant.

30s

22 Repeat the EtOH wash step (#21) 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.

23 Remove the sample tubes off the magnetic rack and resuspend the beads by adding 21 µL of Elution Buffer 2 **(EB2)**. Incubate resuspended beads for 00:02:00 at Room temperature .

2m

24 Place the sample tubes back on the magnetic plate and allow beads to pellet. Transfer 20 µL of the supernatant to a fresh 0.2ml PCR tube.

Safe stopping point: samples can be stored at -20 °C overnight

PCR Library Amplification

2h 10m

25 Transfer 10 µL 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).

26 Add 25 µL of

OneTaq 2X Master Mix with Standard Buffer - 100 rxns **New England Biolabs Catalog #**M0482S

to each sample tube.

27 Using a unique Index Primer per sample, add 15 µL of each respective i7 index primer (Index Primer **N001-N012)** to each sample tube. Pipette to mix. Take note of which index was used for each sample.

Note

The 'forward' primer in the PCR step is:
AATGATACGGCGACCACCGAG

The 'reverse' primer (i7 indexing primer) is:
CAAGCAGAAGACGGCATACGAGATxxxxxGTGACTGGAGTTCAGACGTGT
where 'xxxxxx' denotes the barcode used. Any indexing primers that conform to this 'structure' can be used here.

The 8nt i7 index sequences are provided in the table below:

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

i7 Indexes included in the ClickSeq Technologies Primer Box (12 Indexes)

28 Place the sample tubes in a thermocycler using the following PCR cycling program: 19m 30s

- 94 °C 00:01:00 ; 53 °C 00:00:30 ; 68 °C 00:10:00 ;

- [94 °C 00:00:30 ; 53 °C 00:00:30 ; 68 °C 00:02:00] x 12-21 cycles;
- 68 °C 00:05:00 ;
- 4 °C f∞

Note

The number of PCR cycles to perform will depend on the sample type (species, tissue, quality, 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. We recommended starting with 18 cycles when generating libraries from >100ng of high quality RNA and adjusting as needed.

	A	B
	Total RNA Input	**PCR Cycle Number**
	20ng	18-21
	50ng	17-20
	100ng	16-19
	250ng	12-18

Suggested PCR cycle numbers depending upon the amount of RNA provided in Step #1.

Note

In the case of over- or under-cycling, starting at step #25, the protocol may be repeated using the retained 10µl of your sample from step #24. 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.

Final Bead Purification

15m

29 Add 30 µL of thoroughly resuspended SPRIselect reagent kit **Beckman Coulter Catalog #**B23317 to the reaction mix. Mix

well by pipetting. Incubate for 5 min at room temperature.

30 SPRIselect reagent kit **Beckman Coulter Catalog #**B23317 Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 00:05:00 or until the supernatant is completely clear. 5m

31 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.**

> Note
>
> The SPRI beads discarded at this step retain the longer fragment sizes in the final library generated by ClickSeq (>~600nt). These are generally not compatible with clustering on Illumina flowcells, however, these beads/fragments can be washed with 80% EtOH per the usual protocol, eluted with **EB2** and retained for sequencing on longer-read NGS platforms such as Oxford Nanopore Technologies flowcells.

32 Add 15 µL of thoroughly resuspended SPRI beads to the retained supernatant from step #31. Mix well by pipetting. Incubate for 00:05:00 at Room temperature . 5m

33 Pellet beads by placing the sample tubes on a magnetic rack. Allow the beads to collect for 00:05:00 or until the supernatant is completely clear. 5m

34 Leaving the sample tubes on the magnetic rack, discard clear supernatant. Take care to not disturb the pelleted beads.

35 Leaving the sample tubes on the magnetic rack, wash pelleted beads by adding 200 µL of freshly prepared 80% EtOH. Do not resuspend beads. After 00:00:30 incubation remove and discard the supernatant. 30s

36 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.

37 Remove the sample tubes off the magnetic rack and resuspend the beads by adding 18 µL of Elution Buffer 2 **(EB2)**. Incubate resuspended beads for 00:02:00 at Room temperature .

2m

38 Place the sample tubes back on the magnetic plate and allow beads to pellet. Transfer 17 µL of the supernatant to a fresh tube.

39 Samples are now ready for quality control, quantification, pooling, and sequencing.

Safe stopping point: samples can be stored at -20 °C

Note

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 additional optional steps protocol.

[Optional] Agarose Gel DNA Extraction Protocol (if tighter fragment size distribution is required)

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.

Safe stopping point: samples can be stored at -20 °C

Protocol references

CITATION

Elrod ND, Jaworski EA, Ji P, Wagner EJ, Routh A (2019). Development of Poly(A)-ClickSeq as a tool enabling simultaneous genome-wide poly(A)-site identification and differential expression analysis..

LINK

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