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InDrop Library Preparation - TruDrop (modified V2) Detailed

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ABSTRACT

This protocol details the library preparation process of dual-indexed, NovaSeq-compatible, single-cell RNA-Seq libraries from the inDrop V2 platform. The final library structure combines the TruSeq sequencing primers and indices from standard illumina libraries with the inDrop V2 library features such as the cell barcodes, unique molecular identifier, and transcript. As such it is referred to TruSeq-inDrop (TruDrop).

DOI

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PROTOCOL CITATION

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Southard-Smith, A.N., Simmons, A.J., Chen, B. et al. Dual indexed library design enables compatibility of in-Drop single-cell RNA-sequencing with exAMP chemistry sequencing platforms. BMC Genomics 21, 456 (2020). https://doi.org/10.1186/s12864-020-06843-0

KEYWORDS

inDrop, single cell, scRNA-Seq, dual-indexed

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30120

GUIDELINES

This version of the inDrop protocol requires a moderate to high level of proficiency and should be the only thing really planned for the day once you start it. As soon as an incubation stops you should be proceeding to the next step. This protocol has been optimized to minimize sample down time and plastic transfers to avoid loosing sample to the sides of tubes and pipette tips. Prepare all reagents and consumables prior to starting or between incubations. Managing this balance is critical to increased library diversity yield. It is recommended that a maximum of 6 samples be prepped at a time. All work should be preformed in an RNase free environment. Clean all surfaces (including tube racks and pipettes) with RNaseZap. Pipette tips used should always be filter tips. When thawing reagents always mix by pipetting until no lines of schlieren (i.e. mixing lines) are observed. All reagents and sample tubes should be handled **On ice** .

MATERIALS TEXT

MATERIALS

NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 20 rxns **New England Biolabs Catalog #E6111S**

0.45µm, 2 mL Spin-X Centrifuge tube **Sigma Aldrich Catalog #CLS8162**

HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #KK2601**

Qubit™ Assay Tubes **Invitrogen - Thermo Fisher Catalog #Q32856**

Exonuclease I (E.coli) - 15,000 units **New England Biolabs Catalog #M0293L**

20X EvaGreen **Biotium Catalog #31000**

HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2040S**

Microseal® 'B' Adhesive Seals **BioRad Sciences Catalog #MSB-1001**

Agencourt AMPure XP SPRI beads **Beckman Coulter Catalog #A63881**

Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #Q32852**

DNA LoBind Tube 1.5ml **Eppendorf Catalog #022431021**

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32852**

RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #10777019**

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977015**

PCR Plate, 96-well, non-skirted, black lettering **Thermo Fisher Catalog #AB0600L**

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RNA Fragmentation Reagents **Thermo Fisher Catalog #AM8740**

FastDigest Buffer (10X) **Thermo Fisher Catalog #B64**

Qubit™ 1X dsDNA HS Assay Kit **Thermo Fisher Catalog #Q33230**

PrimeScript™ Reverse Transcriptase **Takarabio Catalog #2680B**

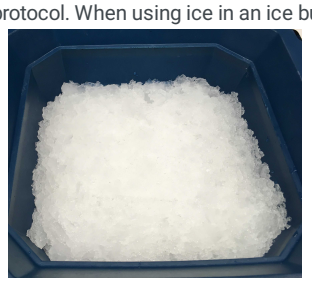
MagJET Separation Rack 12 x 1.5 mL tube **Thermo Fisher Scientific Catalog #MR02**

DNA LoBind Tubes 0.5 mL PCR clean colorless **Eppendorf Catalog #022431005**

PCR-Cooler 0.2 mL **Eppendorf Catalog #022510525**

Primers:

TruDrop Primers.xlsx

You will also need an ice bucket, pipets, a very clean PCR machine. A 96 well block of aluminum is highly recommended for maintaining chilled contact with PCR tubes (available from Light Labs cat no: A-7079) and preventing ice/water from entering them. Always use DNA LoBind tubes for all steps possible in this protocol. When using ice in an ice bucket make sure to pack the ice before using it:  →  . This allows for more contact of tubes with the ice, and the ice has a lower chance of entering the tubes when melting.

Un-packed ice

packed ice

BEFORE STARTING

Prepare the following using RNase and DNase free reagents:

RNA Elution Buffer (RE Buffer)

10 Milimolar (mM) Tris-HCl, pH **7.5**

0.1 Milimolar (mM) EDTA

For **5 mL** :

1 Molarity (M) Tris-HCl pH **7.5** :	**50 µl**
0.5 Molarity (M) EDTA	**10 µl**
RNAse-free H_2O	**4940 µl**

Aliquot into nuclease-free microfuge tubes. RE buffer can be stored at **Room temperature** .

DNA Elution Buffer (DE Buffer)

10 Milimolar (mM) Tris-HCl pH **8.0**

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[M]**0.1 Milimolar (mM)** EDTA

For **5 mL** :

[M]**1 Molarity (M)** Tris-HCl, pH**8.0**	**50 µl**
[M]**0.5 Molarity (M)** EDTA	**10 µl**
RNase-free water	**4940 µl**

Aliquot into nulcease-free microfuge tubes. DE Buffer can be stored at **Room temperature** .

[M]**80 % volume Ethanol**

Combine 8 volumes of ethanol with 2 volumes of nuclease-free water, respectively. Always measure the volumes of both ethanol and water rather than pouring the reagents into a graduated vessel. It is recommended to prepare 15 mL of this at the start of or early on in day 1 and use this throughout day 1 and day 2 until more needs to be prepared on Day 2. ***This reagent is only good for 2 days. If it has been more than 36 hrs since it was prepared fresh reagent should be prepared. I.E. If the prep goes into a 3rd day you will need to make more!**

PE1 and PE2 primer mixes.

Resuspend PE1 and PE2 primers at [M]**100 Micromolar (µM)** in DE Buffer. Dilute all indexing primers (PE1 and PE2) to [M]**10 Micromolar (µM)** (PE2-N6 should be 100 uM starting concentration). Then mix PE1 primer with its corresponding PE2 primer in a 1:1 ratio. The primer mix of PE1 and PE2 should thus be [M]**5 Micromolar (µM)** for each primer

AMPure XP Beads

Aliquot AMPure XP beads in **2 mL** nuclease-free tubes. Store at **4 °C** and bring to **Room temperature** the day of use. Our current longest test show that AMPure beads are stable at **Room temperature** for 2 weeks. Always vortex the AMPure XP beads before using them to ensure they are fully resuspended.

2x KAPA HiFi HotStart Readymix

Prepare 300-310 uL aliquots of the readymix. These should then be stored at **-20 °C**

Before starting on Day 1 do the following:

- Cool bench-top microcentrifuge to **4 °C** .
- Adjust a heat block to **37 °C** .
- It is generally recommended to plan out which indexes will be used for each sample that will be sequenced together prior to starting. This is not 100% necessary though and can be done during the RT incubation on Day 2 if needed.

Oligo Removal 1h 30m

1 **This marks the start of Day 1:**
Before starting do the following:

- Cool bench-top microcentrifuge to **4 °C** .
- Adjust a heat block to **37 °C** .
- It is generally recommended to plan out which indexes will be used for each sample that will be sequenced together prior to starting. This is not 100% necessary though and can be done during the RT incubation on Day 2 if needed.

2

For each sample pre-chill 1 **1.5 mL** DNA LoBind Tube and 1 Spin-X Column + Tube **On ice** . Move a 2 mL aliquot

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of AMPure XP beads to **Room temperature** to start equilibrating. If a heat

3 If necessary, thaw samples on ice.

4 While samples are thawing, visually estimate the volume of each sample. Record this value

5 For every 70 uL of post-RT material prepare 100 uL of Digest mix as follows:
Component:

- Ultrapure RNase/DNase free H_2O **86 µl**
- 10X Fast Digest Buffer **9 µl**
- Exonuclease I **5 µl**

While the samples are thawing mix the H2O and digest buffer. Then add and mix the Exonuclease I to the Digest mix while the sample are spinning in step 4.

6 Centrifuge tubes at **4 °C** for **00:05:00** at **16000 x g** to pellet residual HFE (oil).

7 Adjust the pipette to the estimated sample volume and then block pipette tip with digest mix prior to using it to transfer the sample.

8 Without drawing up HFE-7500 oil from the bottom of the tube, carefully transfer the sample to a Costar Spin-X column.

Once the sample has been drawn into the pipette tip carefully adjust the pipette volume to the exact sample volume. Record this volume. Then eject the sample onto the column. Eject the sample directly above the filter column. Do not let the sample run down the sides of the filter column.

9 Repeat steps 7 — 8 for all samples.

10 Add **100 µl** of digest mix to the filter column for every **70 µl** of sample. Recommended to record the volume added.

- Example calculation: 70 uL sample / 0.7 = 100 uL of digest to be added.

Do not stick the pipette tip into the sample volume. Pipette the digest mix out directly above the sample.

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11 Centrifuge filter column and collection tube at **4 °C** for **00:05:00** at **16000 x g** to pass sample through the filter column.

11.1 During this spin use the previously measured sample volume and digest mix volume to calculate the total volume

12 Place the samples back **On ice** . Adjust the pipet tip to the total volume. Block tip with digest mix. Carefully transfer the sample from the collection tube to 1.5 mL DNA LoBind tube.

Once the sample has been drawn into the pipette tip carefully adjust the pipette volume to the exact sample volume. Record this volume. Then eject the sample into the 1.5mL DNA LoBind tube. Eject the sample at the bottom of the tube. Do not let the sample run down the sides of the tube.

13 Incubate samples at **37 °C** for **00:30:00** .

During this incubation place the 10X Second Strand Synthesis Reaction Buffer (10X SSS buffer) **On ice** to start thawing.

13.1 During the incubation calculate the volume of AMPure XP Beads that will be used in the following 0.8x AMPure purification (AMPurification).

Example: (**165 µl** sample volume) x 0.8 = **132 µl** AMPure XP Beads

14 Vortex the AMPure bead aliquot to resuspend AMPure beads (5-10 seconds is sufficient).

15 Add the appropriate volume of AMPure Beads to each sample and mix by pipetting until no mixing lines are observed (use the same tip that was used to add the AMPure beads to mix the sample). DO NOT VORTEX THE SAMPLE.

16 Incubate each sample/bead mixture at **Room temperature** for **00:05:00** .

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17 Place each sample tube on a magnetic tube rack for **00:02:00** at **Room temperature** or until the beads have fully separated from the supernatant.

Magnetic Stand
Magnetic Stand

Thermo Scientific **MR02**

Any magnetic rack that fits your tubes will suffice.

18 Carefully aspirate the supernatant without removing/disturbing the bead pellet.

While doing this, slowly pipette from the side of each tube that is opposite the bead pellet. Do not touch the beads with the pipette tip.

19 Wash the Bead pellet with **[M]80 % volume** ethanol twice at **Room temperature** .

19.1 For a single sample: Keeping the tube on the magnet rack gently wash the beads with **400 µl** of **[M]80 % volume** ethanol. Rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet.

Pipette the ethanol onto the opposite side of the tube from the beads so as to not greatly disturb the bead pellet. If some beads do not passing from one side of the tube to the other this can sometimes be remedied by taping on the side of the tube where the beads are stuck. If this does not work it is recommended to use the beads that do pass to the other side of the tube to loosen the beads that are stuck by slowly moving the tube around on the magnet such that the beads that were stuck are closest to the magnet.

19.2 Repeat Step 19.1 for each sample.

19.3 Again rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet. All tubes should now be in their original orientation.

19.4 Use a pipette to aspirate and discard the Ethanol. Pipette slowly on the opposite side of the tube from the bead pellet. Again do not disturb the bead pellet with the pipette tip.

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19.5 Repeat steps 19.1 - 19.4 to for the second [M]**80 % volume** ethanol wash.

20 After the second ethanol wash quick-spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect any residual ethanol at the bottom of the tube.

Mini-centrifuge
Centrifuge

Fisher S67601B

Any standard mini centrifuge with adapters for different tube sizes will suffice

21 Place the tubes back on the magnet rack and then use a P200 or smaller pipette to remove any residual ethanol from the bottom of the tube. Do not touch the bead pellet!

22 Leaving the tubes open on the magnetic tube rack allow each bead pellet to air dry (normally 5-10 min) at **Room temperature** . While samples are drying get out 1 PCR tube for each sample (do not use strip tubes or plates) and pre-chill on the 96 well aluminum block **On ice** .

Place a cover over the tubes to prevent dust from falling in them. The pellet will need to dry for at least 5 min. The bead pellet will be a matte black when it is dry and potentially starting to show 1 or 2 cracks. Do not allow the bead pellet to become completely cracked as this will reduce elution efficiency. It is completely normal for some sample to take longer to dry than others (usually larger samples take longer to dry).

23 Once a sample is dry add **17 µl** Ultrapure DNase/RNase free H_2O to just that dry sample and place on a normal tube rack at **Room temperature** .

24 Gently flick the bottom of the tube to re-suspend the bead pellet in the H_2O.

Avoid flinging the solution onto the top half of the tube. Avoid flinging the solution onto the inside of the lid. DO NOT VORTEX!

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25 Incubate the sample at **Room temperature** for **00:05:00** on a normal tube rack. Proceed to step 26 while waiting.

RNA/DNA will not elute if samples are kept cold during this step.

26 While the samples are eluting, mix the thawed 10X SSS buffer by pipetting and add **2 µl** of 10X SSS buffer to each chilled PCR tube.

27 Spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect everything at the bottom of the tube.

28 Place the tube on the magnet rack for about **00:00:10** to pellet the beads on the side of the tube.

If not all of the beads pellet against the magnet that is okay. This is caused by either high protein content/debris left over from the encapsulation or a small amount of residual ethanol. The beads can be removed in the following step.

29 Use a P20 pipette to transfer the supernatant of each sample to its respective chilled PCR tubes and mix by pipetting. If the sample volume is less than 17 uL adjust the volume of the sample up to 17 uL using a P2.

Carrying the magnetic AMPure beads forward should be avoided. Residual AMPure beads can be removed by pipetting the sample out onto the side of the 1.5mL DNA LoBind tube directly above the magnet. Slowly pipette the solution up and down 2-3 times to dislodge any beads stuck to the pipette tip. Allow the beads to pellet against the top of the magnet. Then slowly draw the supernatant back into that same pipette tip while being careful to avoid drawing up any beads that may be present. Throughout this entire process USE A SINGLE PIPETTE TIP PER SAMPLE.

It is very important that the total volume added to the tube is 17 uL of sample in H2O going into SSS.

Second Strand Synthesis (SSS) 3h

30 To each chilled PCR tube add 1 uL of NEBNext Second Strand Synthesis Enzyme mix. Mix the sample by pipetting until few to no mixing lines are observed (10 pipette up and down motions while stirring with the pipet).

31 Start a PCR machine following the below protocol:

16 °C for **02:30:00**

65 °C for **00:20:00**

4 °C hold

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Lid: **50 °C**

Do not place the samples on the PCR machine until the PCR block is at **16 °C** . Once the PCR block temperature is at **16 °C** the samples should be placed on the incubator to start the SSS reaction.

Proceed to preparing the In Vitro Transcription reaction while the SSS reaction is on-going.

In Vitro Transcription (IVT) 16h

32 When the SSS reaction has 50 minutes remaining move the contents of the NEB HiScribe T7 High Yield RNA Synthesis Kit to an ice bucket

to start thawing **On ice** (leave the T7 enzyme at **-20 °C**).

33 When the SSS reaction has 10-15 minutes remaining prepare enough of the following IVT master mix **On ice** for each sample using the NEB HiScribe T7 Kigh Yield RNA Synthesis Kit reagents (mix by pipetting):

Reagent	Volume/Reaction
UltraPure DNase/Rnase-free H2O	**10 µl**
NEB T7 High Yield 10x rxn buffer	**8 µl**
ATP	**8 µl**
CTP	**8 µl**
GTP	**8 µl**
UTP	**8 µl**
NEB T7 High Yield Enzyme Mix	**8 µl**
Pyrophosphatase (NEB M0361S)	**2 µl**

34 When the SSS reaction has completed, quick-spin the samples in PCR tubes for **00:00:02** at **Room temperature** .

35 At **Room temperature** add **60 µl** of IVT master mix to each sample's PCR tube. Mix by pipetting until no mixing lines are observed.

36 Start a PCR machine to the follow the below protocol:

37 °C for **15:00:00**

Lid: **50 °C**

Do not place the samples on the PCR machine until the PCR block is at **37 °C** . Once the PCR block temperature is at **37 °C** the samples should be placed on the incubator to start the SSS reaction.

37 **This marks the start of Day 2:** 5m

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Prepare an ice bucket (pack the ice). **On ice** chill the 96 well aluminum block. Prepare a single 1.5 mL DNA LoBind tube for each sample.

38 Vortex the AMPure bead aliquot to resuspend the AMPure XP beads (5-10 seconds is sufficient for a **2 mL** aliquot).

39 Add the 72 uL of AMPure Beads to each sample and mix by pipetting until no mixing lines are observed (use the same tip that was used to add the AMPure beads to mix the sample). DO NOT VORTEX THE SAMPLE.

40 Incubate each sample/bead mixture at **Room temperature** for **00:05:00** .

41 Place each sample tube on a magnetic tube rack for **00:02:00** at **Room temperature** or until the beads have fully separated from the supernatant.

Magnetic Stand
Magnetic Stand

Thermo Scientific **MR02**

Any magnetic rack that fits your tubes will suffice.

42 Carefully aspirate the supernatant without removing/disturbing the bead pellet.

While doing this, slowly pipette from the side of each tube that is opposite the bead pellet. Do not touch the beads with the pipette tip.

43 Wash the Bead pellet with **[M]80 % volume** ethanol twice at **Room temperature** .

43.1 For a single sample: Keeping the tube on the magnet rack gently wash the beads with **400 µl** of **[M]80 % volume** ethanol. Rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet.

Pipette the ethanol onto the opposite side of the tube from the beads so as to not greatly disturb the bead pellet. If some beads do not passing from one side of the tube to the other this can sometimes be remedied by taping on the side of the tube where the beads are stuck. If this does

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not work it is recommended to use the beads that do pass to the other side of the tube to loosen the beads that are stuck by slowly moving the tube around on the magnet such that the beads that were stuck are closest to the magnet.

During the first Ethanol wash of the AMPurification after IVT it is normal to see a small cloud of white material precipitate out. This can be observed when drawing up and discarding the ethanol in subsequent steps.

43.2 Repeat Step 43.1 for each sample.

43.3 Again rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet. All tubes should now be in their original orientation.

43.4 Use a pipette to slowly aspirate and discard the Ethanol. Draw the ethanol up from the opposite side of the bead pellet. Do not disturb the bead pellet with the pipette tip.

43.5 Repeat steps 19.1 - 19.4 to for the second [M]**80 % volume** ethanol wash.

44 After the second ethanol wash, quick-spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect any residual ethanol at the bottom of the tube.

Mini-centrifuge
Centrifuge

Fisher S67601B

Any standard mini centrifuge with adapters for different tube sizes will suffice

45 Place the tubes back on the magnet rack and then use a P200 or smaller pipette to remove any residual ethanol from the bottom of the tube. Do not touch the bead pellet!

46 Leaving the tubes open on the magnetic tube rack allow each bead pellet to air dry for **00:05:00** at **Room temperature** . While samples are drying get out 1 PCR tube and one 0.5 mL DNA LoBind tube for each sample (do not use strip tubes or plates) and pre-chill **On ice** . Use the 96 well aluminum block for the PCR tubes.

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Place a dust-free cover over the drying tubes to prevent dust from falling into them. All samples should be dry after 5 minutes. On rare occasions a sample may take 6 or 7 minutes to dry.

47 Once a sample is dry, add **20 µl** RNA Elution buffer to just that dry sample and place on a normal tube rack at **Room temperature** .

48 Gently flick the bottom of the tube to re-suspend the bead pellet in the H_2O.

Avoid flinging the solution onto the top half of the tube. Avoid flinging the solution onto the inside of the lid. DO NOT VORTEX!

49 Incubate the sample at **Room temperature** for **00:05:00** on a normal tube rack. During this time prepare 1 Qubit tube for each sample, 2 Qubit tubes for standards, and one 2 mL tube for preparing the Qubit reagents.

RNA/DNA will not elute if samples are kept cold during this step.

50 Spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect everything at the bottom of the tube.

Spinning for longer than 2 seconds here can result in AMPure beads getting stuck at the bottom of the tube at step 51.

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

51 Place the tube on the magnet rack for about **00:00:10** to pellet the beads on the side of the tube.

If not all of the beads pellet against the magnet that is okay. They can be removed in the following step.

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52 Use a P20 pipette to transfer the supernatant (**20 µl**) of each sample to its respective chilled 0.5 mL DNA LoBind tube. Using the same pipette tip transfer **9 µl** of the sample to it's respective chilled PCR tube. Repeat this process for the remaining samples. The PCR tube is now the sample tube. The 0.5 mL DNA LoBind tube is now the backup.

Carrying the magnetic AMPure beads forward should be avoided. Residual AMPure beads can be removed by pipetting the sample out onto the side of the 1.5mL DNA LoBind tube directly above the magnet. Slowly pipette up and down 2-3 times, and then allow the beads to cluster on the magnet for 2-3 seconds. Then slowly draw the supernatant back into that same pipette tip while being careful to avoid drawing up any beads that may be present.

53 Use 2 uL from the backup tube to measure the RNA concentration of the sample via Qubit using the Qubit RNA HS assay kit. At this point it is also optional to use 1 uL of the sample for evaluation using a BioAnalyzer RNA Pico Chip Assay.

The Qubit RNA HS reagent from the Qubit RNA HS assay kit is very light sensitive. Only use it with the lights off.

RNA yields are highly dependent on the types of cells and cell viability in the sample during encapsulation.
For mouse/human intestinal epithelium cells:
0-20 ng/uL RNA is low.
20-60 ng/uL of RNA is normal.
60+ ng/uL of RNA is high.
If the RNA yield exceeds 85 ng/uL then the sample should be diluted to 80 ng/uL before proceeding to fragmentation.
If the Qubit output is "TL" (i.e. Too Low) a library can still sometimes be prepared, but all of the sample (**18 µl**)should be carried forwards (**18 µl**).

If samples will be further processed another day this is a safe pause point. Samples can be stored at **-80 °C** .

Fragmentation 1h 10m

54 According to 1CellBio: if the samples were run on a BioAnalyzer and the average size <800 bp fragmentation then it is not recommendd to fragment the library, and one can proceed Reverse Transcription using random hexamers.

If samples were thawed from backup tubes transfer **9 µl** to a PCR tube. Top off any missing volume with RNA Elution Buffer. Otherwise proceed to step 55.

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55 Set up a thermal cycler to follow the below protocol:

70 °C hold

lid: **105 °C**

56 Prepare the following Fragmentation Stop mix in a DNA LoBind tube and keep at room temperature:

Component	Volume per Reaction
Ultrapure DNase/RNase-free H2O	9 uL
10x Fragmentation Stop Solution	1 uL
AMPure Beads	16 uL
Total	26 uL

57 For each sample Pre-chill a single 1.5mL DNA LoBind tube **On ice** . Also pre-set a P200 to 26 uL. Steps 58-61 should be completed as quickly as possible after this.

58 Warning: Wait to do this step until the thermal cycler is at **70 °C** . Add 1 uL of 10X RNA fragmentation reagent to each sample. Mix by pipetting (5 times) and stirring. Immediately place back on chilled 96 well aluminum block.

59 Once the fragmentation reagent has been added to all samples, place all samples on the pre-heated thermal cycler for 1-2 minutes. Refer to the below table for guidance with regards to fragmentation duration.

IVT yield ranges (ng/uL)	Example fragmentation durations for intestinal epithelium cell libraries (m:ss)
0-2	0:00 to 0:20
2-10	0:20 to 1:00
10-40	1:00 to 1:30
40-60	1:30 to 1:45
60-80	1:40 to 1:50
80+	1:50 to 2:00

60 After fragmentation immediately place all samples on the chilled 96 well aluminum block.

61 Add 26 uL of Fragmentation Stop mix to each sample and transfer that sample to its pre-chilled 1.5 mL DNA LoBind tube.

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For minimal sample loss Use a P200 pipette to add 26 uL of Fragmentation Stop mix to a single sample. Using the same pipette tip immediately transfer that sample to the 1.5 mL tube. Mix by pipetting until no mixing lines are observed. Repeat this for all samples (use a new pipette tip for each sample).

62 Incubate the samples on ice for **00:05:00** . At this time place aliquots of the 1x PrimeScript RT buffer, the **100 Micromolar (µM)** TruDrop PE2-N6 primer, and dNTPs **On ice** to start thawing.

63 Place each sample tube on a magnetic tube rack for **00:02:00** at **Room temperature** or until the beads have fully separated from the supernatant.

Magnetic Stand
Magnetic Stand

Thermo Scientific **MR02**

Any magnetic rack that fits your tubes will suffice.

64 Use a P200 pipette to carefully aspirate the supernatant without removing/disturbing the bead pellet.

While doing this, slowly pipette from the side of each tube that is opposite the bead pellet. Do not touch the beads with the pipette tip.

65 Wash the Bead pellet with **80 % volume** ethanol twice at **Room temperature** .

65.1 For a single sample: Keeping the tube on the magnet rack gently wash the beads with **400 µl** of **80 % volume** ethanol. Rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet.

Pipette the ethanol onto the opposite side of the tube from the beads so as to not greatly disturb the bead pellet. If some beads do not pass from one side of the tube to the other, this can sometimes be remedied by taping on the side of the tube where the beads are stuck. For this AMPurification in particular speed is generally prioritized over passing all beads across the tube so one should proceed to step 65.2. If one would still like to have stuck beads pass across the tube then follow the instructions below.

If this does not work it is recommended to use the beads that do pass to the other side of the tube to loosen the beads that are stuck by slowly moving the tube around on the magnet such that the beads that were stuck are closest to the magnet. At this point, slightly moving the tube up and

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down with some rotation can then sometimes dislodge the beads that are stuck. The tube can then be rotated 180° again to pass all other beads across the tube. If some beads are still stuck after this it is generally recommended to proceed anyways. It is not essential for all beads to pass across the tube though it is recommended.

65.2 Repeat Step 65.1 for each sample.

65.3 Again rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet. All tubes should now be in their original orientation.

65.4 Use a pipette to slowly aspirate and discard the Ethanol for each sample. Draw the ethanol up from the opposite side of the bead pellet. Do not disturb the bead pellet with the pipette tip.

65.5 Repeat steps 65.1 - 65.4 to for the second [M] **80 % volume** ethanol wash.

66 After the second ethanol wash, quick-spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect any residual ethanol at the bottom of the tube.

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

67 Place the tubes back on the magnet rack and then use a P200 or smaller pipette to remove any residual ethanol from the bottom of the tube. Do not touch the bead pellet!

68 Leaving the tubes open on the magnetic tube rack allow each bead pellet to air dry for **00:05:00** at **Room temperature** . While samples are drying, do the following:

- Set up and start a thermal cycler with the following protocol:

  65 °C hold

  Lid: **105 °C**

- Pre-chill 1 PCR tube for each sample **On ice** . Use the 96 well aluminum block for the PCR tubes.
- If possible, add 1 uL of dNTP's with 2 uL of TruDrop PE2-N6 primer in the PCR tube (otherwise this can be done

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later).

Place a dust-free cover over the tubes to prevent dust from falling into them. All samples should be dry after 5 minutes. On rare occasions a sample may take 6 or 7 minutes to dry.

Always mix the PE2-N6 primer and dNTP's by pipetting before using it or aliquoting it out. This should be done until no mixing lines are observed.

69 Once a sample is dry add **8 µl** RNA Elution buffer to just that dry sample and place on a normal tube rack at **Room temperature** .

70 Gently flick the bottom of the tube to re-suspend the bead pellet in the RNA Elution buffer.

Avoid flinging the solution onto the top half of the tube. Avoid flinging the solution onto the inside of the lid. DO NOT VORTEX!

71 Incubate the sample at **Room temperature** for **00:05:00** on a normal tube rack. During this time perform the following:

- finish adding **1 µl** of dNTPs and **2 µl** of TruDrop PE2-N6 primer to the pre-staged PCR tubes.
- Start preparing the following RT mastermix in a DNA LoBind Tube (only add the H2O and RT buffer):

Reagent	Volume Per Reaction
Ultrapure DNase/RNase-free H2O	2 µL
5x PrimeScript RT buffer	4 µL
RNase OUT (40 U/µL)	2 µL
PrimeScript RT enzyme (200 U/µL)	1 µL
total	9 µL

RNA/DNA will not elute if samples are kept cold during this step.

Always mix the PE2-N6 primer, dNTP's, and RT buffer by pipetting before using it or aliquoting it out. This should be done until no mixing lines are observed.

72 Spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect everything at the bottom of the tube.

Spinning for longer than 2 seconds here can result in AMPure beads getting stuck at the bottom of the tube at step

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51.

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

73 Place the tube on the magnet rack for about **00:00:10** to pellet the beads on the side of the tube.

If not all of the beads pellet against the magnet that is okay. They can be removed in the following step.

74 Use a P20 pipette to transfer the supernatant (**8 µl**) of each sample to its respective chilled PCR tube. Pipette up and down (5x) to mix the sample with the primer and dNTPs.

Carrying the magnetic AMPure beads forward should be avoided. Residual AMPure beads can be removed by pipetting the sample out onto the side of the 1.5mL DNA LoBind tube directly above the magnet. Slowly pipette up and down 2-3 times, and then allow the beads to cluster on the magnet for 2-3 seconds. Then slowly draw the supernatant back into that same pipette tip while being careful to avoid drawing up any beads that may be present.

2nd Reverse Transcription 2h 30m

75 If it has not been done yet, start preparing the following RT mastermix in a DNA LoBind Tube (only add the H2O and RT buffer):

Reagent	Volume Per Reaction
Ultrapure DNase/RNase-free H2O	2 µL
5x PrimeScript RT buffer	4 µL
RNase OUT (40 U/µL)	2 µL
PrimeScript RT enzyme (200 U/µL)	1 µL
total	9 µL

Always mix the RT buffer by pipetting before using it or aliquoting it out. This should be done until no mixing lines are observed.

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76 Incubate all samples at **65 °C** for **00:03:00** on the thermal cycler set up in step 68. During this time finish making the RT master mix (step 75, add the RNase OUT and PrimScript RT enzyme). Mix by pipetting.

77 Remove all samples form the thermal cycler and cool on ice (use the chilled 96-well block for about 5-10 seconds) before adding 9 uL of RT master mix to each sample. Using the same pipette tip that was used to add the RT mix, mix the sample by pipetting.

78 Start a thermal cycler following the below protocol:

30 °C for **00:10:00**

42 °C for **01:00:00**

70 °C for **00:15:00**

4 °C hold

lid: **105 °C**

Place the samples on the thermal cycler heat-block when it reaches **30 °C** . During this time prepare two 1.5 mL DNA LoBind tubes for each sample. Pre-chill one of the two **On ice** . Keep the other one at room temperature. If more **[M]80 % volume** ethanol will be needed now is a great time to prepare it.

During this time if the sample indices have not already been chosen for each sample that should be done here. The PCR reactions after the RT cannot be completed without knowing which samples will have which index pairs. For assistance in picking which indices can be used together it is recommended to follow the indexing guidelines in the Illumina document "index-adapters-pooling-guide-1000000041074-08" (specifically the section: "IDT for Illumina-TruSeq UD Indexes").

Further indexing guidelines can be found in the Sequencing section (step 130) of this protocol.

79 Place all samples on the chilled 96 well block **On ice** . Add **30 µl** of Ultrapure DNase/RNase-free H_2O to each sample.

80 Use a P200 pipette to add **35 µl** of Ampure XP beads to a single sample. Using the same pipette tip, transfer the sample to a room temperature 1.5 mL DNA LoBind Tube and pipette up and down until no mixing lines are observed. DO NOT VORTEX.

80.1 Repeat step 93 for each sample.

81 Incubate each sample/bead mixture at **Room temperature** for **00:05:00** .

During this time do the following:

- If needed, prepare more **[M]80 % volume** ethanol. Otherwise:
- **On ice** start thawing the TruDrop PE1/PE2 indexed **[M]5 Micromolar (µM)** primer mixes that will be used for

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each sample.
- Finish preparing a 1.5 mL DNA LoBind tube for each sample and pre-chill on ice.

82 Place each sample tube on a magnetic tube rack for **00:02:00** at **Room temperature** or until the beads have fully separated from the supernatant.
During this time do the following:
- **On ice** start thawing the TruDrop PE1/PE2 indexed **5 Micromolar (µM)** primer mixes that will be used for each sample.

At the end of the 2 minutes the pellet can still look slightly diffuse. This is normal. Proceed to the next step anyways. The post-2nd-RT AMPurification can be very finicky. The diffuse nature of the beads is due to either the high glycerol concentration from the RT, or the large amount of protein from the RT, or both.

Magnetic Stand
Magnetic Stand

Thermo Scientific **MR02**

Any magnetic rack that fits your tubes will suffice.

83 Carefully aspirate the supernatant without removing/disturbing the bead pellet.

While doing this, use a P200 pipette to slowly pipette from the side of each tube that is opposite the bead pellet. Do not touch the beads with the pipette tip. Do not aspirate any beads! If beads are removed by accident they can be recovered as follows:
- Hold the magnet rack such that the side of the tube at the top of the magnet is horizontal.
- Slowly pipette the entire solution out onto the top of the magnet making sure the supernatant does not contact the bead pellet.
- Slowly draw the supernatant up into the pipette, leaving behind any small amount of beads pulled out of solution on the side of the tube. If a 1-2 uL drop of supernatant is left behind that will not be a major problem.

84 Wash the Bead pellet with **80 % volume** ethanol twice at **Room temperature** .

84.1 For a single sample: Keeping the tube on the magnet rack gently wash the beads with **400 µl** of **80 % volume** ethanol. Rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet.

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Pipette the ethanol onto the opposite side of the tube from the beads so as to not greatly disturb the bead pellet. Then, rotate the tube. If some beads do not passing from one side of the tube to the other this can sometimes be remedied by taping on the side of the tube where the beads are stuck. If this does not work it is recommended to use the beads that do pass to the other side of the tube to loosen the beads that are stuck by slowly moving the tube around on the magnet such that the beads that were stuck are closest to the magnet. At this point, slightly moving the tube up and down with some rotation can then sometimes dislodge the beads that are stuck. The tube can then be rotated 180° again to pass all other beads across the tube. If some beads are still stuck after this it is generally recommended to proceed anyways. It is not essential for all beads to pass across the tube though it is recommended.

During the first ethanol wash of the AMPurification after 2nd RT it is very normal for all of the beads to not pass across the tube when rotated on the magnet rack. The best way to avoid this is to rotate the tube ASAP after adding the first ethanol wash. It may take more tapping/moving/rotating the tube than normal to pass all beads across.

84.2 Repeat Step 97.1 for each sample.

84.3 Again rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet. All tubes should now be in their original orientation.

84.4 Use a pipette to slowly aspirate and discard the Ethanol. Draw the ethanol up from the opposite side of the bead pellet. Do not disturb the bead pellet with the pipette tip.

84.5 Repeat steps 97.1 - 97.4 to for the second [M]**80 % volume** ethanol wash.

85 After the second ethanol wash, quick-spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect any residual ethanol at the bottom of the tube.

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

86 Place the tubes back on the magnet rack and then use a P200 or smaller pipette to remove any residual ethanol from

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the bottom of the tube. Do not touch the bead pellet!

87 Leaving the tubes open on the magnetic tube rack, allow each bead pellet to air dry for **00:02:00** at **Room temperature** . If a single 1.5 mL DNA LoBind tube has not yet been pre-chilled for each sample, prepare those now.

Place a dust-free cover over the drying tubes to prevent dust from falling into them. All samples should be dry after 2—2:30 minutes. At this step, it is generally recommended to just elute all samples here after 2 minutes. Allowing samples to over-dry here will result in the pellet becoming cracked very quickly, and them not being able to be fully re-suspended during elution.

88 Once a sample is dry, add **40 µl** DNA Elution buffer to just that dry sample and place on a normal tube rack at **Room temperature** .

89 Gently flick the bottom of the tube to re-suspend the bead pellet in the DNA Elution buffer.

Avoid flinging the solution onto the top half of the tube. Avoid flinging the solution onto the inside of the lid. DO NOT VORTEX!

90 Incubate each sample/bead mixture at **Room temperature** for **00:05:00** .
While samples are eluting:

- Get out 1 PCR tube for each sample (do not use strip tubes or plates) and pre-chill **On ice** . Use the 96 well aluminum block for the PCR tubes.
- Start thawing a 300 uL aliquot of 2x KAPA HiFi HotStart **On ice** .

91 Spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect everything at the bottom of the tube.

Spinning for longer than 2 seconds here can result in AMPure beads getting stuck at the bottom of the tube at step 51.

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Mini-centrifuge
Centrifuge

Fisher S67601B

Any standard mini centrifuge with adapters for different tube sizes will suffice

92 Place each sample tube on a magnetic tube rack for **00:00:15** at **Room temperature** , or until the beads have fully separated from the supernatant.

For the RT Ampurification it is normal to have a thin film of beads that remain and continue to coat the bottom of the tube when placed against the magnet. As long as a distinct small pellet of beads is observed close to the magnet it is okay to proceed to step 106.

93 Use a P20 pipette to transfer the supernatant (**40 µl**) of each sample to its respective chilled 1.5 mL DNA LoBind tube. Using the same pipette tip transfer **20 µl** of the sample to it's respective chilled PCR tube. Repeat this process for the remaining samples. The PCR tube is now the sample tube. The 1.5 mL DNA LoBind tube is now the pre-PCR backup.

Carrying the magnetic AMPure beads forward should be avoided. Residual AMPure beads can be removed by pipetting the sample out onto the side of the 1.5mL DNA LoBind tube directly above the magnet. Slowly pipette up and down 2-3 times, and then allow the beads to cluster on the magnet for 2-3 seconds. Then slowly draw the supernatant back into that same pipette tip while being careful to avoid drawing up any beads that may be present.

This can be a pause point. It is highly recommended that all samples proceed with library preparation to finish preparing the final library. However, if necessary the tubes at this stage can be frozen at **-80 °C** until the library preparation can continue.

Diagnostic qPCR 2h 30m

94 Mix the thawed 2x KAPA and all of the TruDrop primer mixes to be used by pipetting until no mixing lines are observed or at least by pipetting up and down 5x. Next, place a 96 well PCR Plate into a pre-frozen (**-20 °C**) PCR-Cooler rack.

Citation: Austin Southard-Smith, Alan J Simmons, Ken Lau (08/03/2021). InDrop Library Preparation - TruDrop (modified V2) Detailed. https://dx.doi.org/10.17504/protocols.io.9ngh5bw

95 Prepare the following qPCR mixture on ice:

Reagent	Volume per reaction
Ultrapure DNase/RNase-free H2O	13 µL
2x KAPA HiFi HotStart PCR Mix	20 µL
20x Eva Green Dye	2 µL
Total	35 µL

96 Decide and record which sample will be PCR'ed in which sample well. It is recommended to space your samples so that none are located on the border of the plate. Example: We always use column 4 starting at row B and ending at row G. Add 35 uL of qPCR mixture to each well that will contain a sample.

97 Add 4 uL of the TruDrop PE1/PE2 primer mixture that corresponds to each sample to the well of the PCR plate where that sample will be located.

98 Add 1 uL of a single sample to it's corresponding well in the PCR plate. Make sure to pipette out into the liquid in each well and not onto the side of the plate.

98.1 Repeat step 111 for each sample

99 Seal the plate with a MicroSeal (BioRad MSB-100). Use the smooth side of the paper protector to press down around the edges of the wells containing the samples and around the edges of the plate.

100 Spin the qPCr Plate at **600 rpm, 4°C, 00:01:00** to collect everything at the bottom of the well(s) and remove any bubbles.

101 Set up a qPCR machine with the following protocol, load the plate in the machine and start the PCR machine:

1) **98 °C** for **00:02:00**
2) **98 °C** for **00:00:20**
3) **55 °C** for **00:00:30**
4) **72 °C** for **00:00:40**
5) Go to 2) once (a total of two cycles)
6) **98 °C** for **00:00:20**
7) **65 °C** for **00:00:30**
8) **72 °C** for **00:00:40** with fluorescent read
9) Go to 6) 23 times (a total of 24 cycles)

Lid: **105 °C**

Citation: Austin Southard-Smith, Alan J Simmons, Ken Lau (08/03/2021). InDrop Library Preparation - TruDrop (modified V2) Detailed. https://dx.doi.org/10.17504/protocols.io.9ngh5bw

Volume: **40 µl**

This PCR normally takes about 1 hour 25 minutes. While the reaction is going the remaining sample (19.5 uL) PCR tubes, primer tubes, and KAPA should be placed on the chilled 96 well aluminum block and then stored in a clean 4oC fridge during the course of the reaction. This is also a good point to replenish the ice in the ice bucket.

102 Set the qPCR threshold within the exponential phase of amplification, but closer to where the signal starts to emerge from the noise to avoid the possibility of over amplification. We normally wil adjust to about 5000 RFU.

103 Correct the resulting Ct value to account for differences in the amount of input material between the qPCR and library PCR. The library input is 19 times higher, and assuming a 100% PCR efficiency, the difference corresponds to log2 19 = 4.25 cycles. For example if the number of cycles determined by qPCR is 2 + 10.27, the required number of PCR cycles for library is 2 + (10.27 — 4.25) = 2 + 6.02 . This would then be rounded to 2 + 6 cycles. The rules we use for rounding here is that any number with a decimal value of 0.4 or more is rounded up to the nearest integer, while any number with a decimal value less that 0.4 is rounded down to the nearest integer. Example: 6.38 would be rounded down to 6 cycles, but 6.42 is rounded up to 7 cycles. Record this rounded value as the 2 + N, where N is the rounded adjusted number of cycles.

Expected result is normally 4-7 cycles depending on the sample.

PCR Amplification

104 Move the 96 well aluminum block with the samples, primer mixes, and KAPA back to the ice bucket.

105 Prepare the following PCR mixture **On ice** :

Reagent	Volume per reaction
Ultrapure DNase/RNase-free H2O	1 uL
2x Kapa HiFi HotStart PCR mix	25 uL
Total	26 uL

106 To each sample add 5 uL of TruDrop PE1/PE2 primer mix to the corresponding sample tube.

107 To each sample add 26 uL of PCR mix to the corresponding sample tube. Mix the sample by pipetting until no mixing lines are observed.

Citation: Austin Southard-Smith, Alan J Simmons, Ken Lau (08/03/2021). InDrop Library Preparation - TruDrop (modified V2) Detailed. https://dx.doi.org/10.17504/protocols.io.9ngh5bw

108 Set up and start a PCR machine to follow the below protocol (wait to place the samples on the machine until the heat block is **95 °C**):

1) **98 °C** for **00:02:00**

2) **98 °C** for **00:00:20**

3) **55 °C** for **00:00:30**

4) **72 °C** for **00:00:40**

5) Go to 2) once (a total of two cycles)

6) **98 °C** for **00:00:20**

7) **65 °C** for **00:00:30**

8) **72 °C** for **00:00:40** with fluorescent read

9) Go to 6) N-1 times (a total of N adjusted cycles).

10) **72 °C** for **00:05:00**

11) hold **4 °C**

Lid: **105 °C**

Volume: **50 µl** To the sample

The value for N was determined as a part of step 116. If working with multiple libraries that require different numbers of cycles, set the thermal cycler to perform the highest number of cycles required, and manually remove individual libraries from the thermal cycler, when they reach the desired cycle number. For example, if library X requires 8 cycles, at the end of the 72°C step of cycle 8 pause the cycler and transfer library X to an ice bucket. Then, resume the PCR program. Once all the samples have undergone the required number of PCR cycles, transfer all tubes back to the thermal cycler for the final step, a 5 min incubation at 72°C.

During the final 5 min extension at 72oC prepare 3 DNA LoBind tubes for each sample. 2 tubes will be kept at **Room temperature** . The final tube should be chilled **On ice** . To one of each sample's room temperature LoBind tubes add 20 uL of AMPure beads (part of this can be done later).

109 When the PCR is finished transfer all samples to the chilled 96-well aluminum block **On ice** . Add **50 µl** of DNA Elution buffer to the PCR product.

110 To a single sample add 60 uL of AMPurebeads. Using the same pipette tip transfer the sample + bead mixture to its empty DNA LoBind tube. Mix by pipetting until no mixing lines are observed. DO NOT VORTEX.

This is the start of a double sided AMPurification. The lower bound is 0.6x (step 110). The upper bound is 0.8x (step 113).

110.1 Do step 123 for all other samples.

111 Incubate each sample/bead mixture at **Room temperature** for **00:05:00** .

During this time do the following:

- If needed, prepare more **80 % volume** ethanol.
- Finish adding 20 uL of AMPure beads to the 2nd set of room temperature tubes that will be used.

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112 Place each sample tube on a magnetic tube rack for **00:02:00** at **Room temperature** . It is important that samples are on the magnet rack for the full 2 minutes.

Magnetic Stand
Magnetic Stand

Thermo Scientific **MR02**

Any magnetic rack that fits your tubes will suffice.

113 The finished library is what is in the supernatant. For a single sample, carefully transfer the supernatant to the second room temperature 1.5 mL DNA LoBind tube containing 20 uL of AMPure beads. Do not carry forward any beads. Using the same pipette tip, mix the sample by pipetting until no mixing lines are observed. The second tube is now the sample tube.

While doing this, use a P200 pipette to slowly pipette from the side of each tube that is opposite the bead pellet. Do not touch the beads with the pipette tip. Do not aspirate any beads! If beads are removed by accident they can be removed as follows:

- Close alltubes and hold the magnet rack such that the side of the tube at the top of the magnet is horizontal.
- Slowly pipette the entire solution out onto the top of the magnet making sure the supernatant does not contact the bead pellet.
- Slowly draw the supernatant up into the pipette, leaving behind any small amount of beads pulled out of solution on the side of the tube. If a 1-2 uL drop of supernatant is left behind that will not be a major problem.

113.1 Repeat step 126 for all remaining samples.

114 Incubate each sample/bead mixture at **Room temperature** for **00:05:00** on a normal tube rack.

115 Place each sample tube on a magnetic tube rack for **00:02:00** at **Room temperature** . It is important that samples are on the magnet rack for the full 2 minutes.

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Magnetic Stand
Magnetic Stand

Thermo Scientific **MR02**

Any magnetic rack that fits your tubes will suffice.

116 The sample is now what is on the beads. Carefully aspirate the supernatant without removing/disturbing the bead pellet.

While doing this, use a P200 pipette to slowly pipette from the side of each tube that is opposite the bead pellet. Do not touch the beads with the pipette tip. Do not aspirate any beads! If beads are removed by accident they can be recovered as follows:

- Hold the magnet rack such that the side of the tube at the top of the magnet is horizontal.
- Slowly pipette the entire solution out onto the top of the magnet making sure the supernatant does not contact the bead pellet.
- Slowly draw the supernatant up into the pipette, leaving behind any small amount of beads pulled out of solution on the side of the tube. If a 1-2 uL drop of supernatant is left behind that will not be a major problem.

117 Wash the Bead pellet with **80 % volume** ethanol twice at **Room temperature** .

117.1 For a single sample: Keeping the tube on the magnet rack gently wash the beads with **400 µl** of **80 % volume** ethanol. Rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet.

Pipette the ethanol onto the opposite side of the tube from the beads so as to not greatly disturb the bead pellet. Then, rotate the tube. If some beads do not passing from one side of the tube to the other this can sometimes be remedied by taping on the side of the tube where the beads are stuck. If this does not work it is recommended to use the beads that do pass to the other side of the tube to loosen the beads that are stuck by slowly moving the tube around on the magnet such that the beads that were stuck are closest to the magnet. At this point, slightly moving the tube up and down with some rotation can then sometimes dislodge the beads that are stuck. The tube can then be rotated 180° again to pass all other beads across the tube. If some beads are still stuck after this it is generally recommended to proceed anyways. It is not essential for all beads to pass across the tube though it is recommended.

117.2 Repeat Step 117.1 for each sample.

Citation: Austin Southard-Smith, Alan J Simmons, Ken Lau (08/03/2021). InDrop Library Preparation - TruDrop (modified V2) Detailed. https://dx.doi.org/10.17504/protocols.io.9ngh5bw

117.3 Again rotate each tube 180°, allowing the beads to re-pellet on the side now closest to the magnet. All tubes should now be in their original orientation.

117.4 Use a pipette to slowly aspirate and discard the Ethanol. Draw the ethanol up from the opposite side of the bead pellet. Do not disturb the bead pellet with the pipette tip.

117.5 Repeat steps 117.1 - 117.4 to for the second [M]**80 % volume** ethanol wash.

118 After the second ethanol wash, quick-spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect any residual ethanol at the bottom of the tube.

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

119 Place the tubes back on the magnet rack and then use a P200 or smaller pipette to remove any residual ethanol from the bottom of the tube. Do not touch the bead pellet!

120 Leaving the tubes open on the magnetic tube rack allow each bead pellet to air dry for **00:02:00** at **Room temperature** . If a single 1.5 mL DNA LoBind tube has not yet been pre-chilled for each sample, prepare those now.

Place a dust-free cover over the drying tubes to prevent dust from falling into them. All samples should be dry after 2—2:30 minutes.

121 Once a sample is dry, add **20 µl** DNA Elution buffer to just that dry sample and place on a normal tube rack at **Room temperature** .

122 Gently flick the bottom of the tube to re-suspend the bead pellet in the DNA Elution Buffer.

Citation: Austin Southard-Smith, Alan J Simmons, Ken Lau (08/03/2021). InDrop Library Preparation - TruDrop (modified V2) Detailed. https://dx.doi.org/10.17504/protocols.io.9ngh5bw

Avoid flinging the solution onto the top half of the tube. Avoid flinging the solution onto the inside of the lid. DO NOT VORTEX!

123 Incubate each sample/bead mixture at **Room temperature** for **00:05:00** .

124 Spin the sample tubes in a mini-centrifuge for **00:00:01** at **Room temperature** to collect everything at the bottom of the tube.

Spinning for longer than 2 seconds here can result in AMPure beads getting stuck at the bottom of the tube at step 51.

Mini-centrifuge
Centrifuge

Fisher **S67601B**

Any standard mini centrifuge with adapters for different tube sizes will suffice

125 Place each sample tube on a magnetic tube rack for **00:00:15** at **Room temperature** , or until the beads have fully separated from the supernatant.

For the RT Ampurification it is normal to have a thin film of beads that remain and continue to coat the bottom of the tube when placed against the magnet. As long as a distinct small pellet of beads is observed close to the magnet it is okay to proceed to step 106.

126 Use a P20 pipette to transfer the supernatant (**20 µl**) of each sample to its respective chilled 1.5 mL DNA LoBind tube. This is the final library.

Carrying the magnetic AMPure beads forward should be avoided. Residual AMPure beads can be removed by pipetting the sample out onto the side of the 1.5mL DNA LoBind tube directly above the magnet. Slowly pipette up and down 2-3 times, and then allow the beads to cluster on the magnet for 2-3 seconds. Then slowly draw the supernatant back into that same pipette tip while being careful to avoid drawing up any beads that may be present.

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127 Use 2 uL from the sample to measure the DNA concentration of the sample via Qubit using the Qubit DNA HS assay kit.

Sample Concentration should be between 1-3 ng/uL.

128 Samples should be stored at **-80 °C** until they can be sequenced.

Sequencing

129 Quantify samples via BioAnalyzer and qPCR prior to sequencing.

130 If sequencing TruDrop libraries in pools alongside higher percentages of other Illumina compatible libraries (70% of the total pool or more is from other Illumina compatible libraries), then one can use each pair of indexes (one i7, and one i5) once per sequencing lane per sequencing run. There are no other limitations on sequencing index usage, provided that a library can be made from the index pair (do not use pair UDI0009). Example: UDI0025 could be used with UDI0001. So long as the indices aren't repeated across multiple libraries in a single sequencing pool. This option is recommended so as to not run out of certain primer pair before others.

Disclaimer: Our operating range is 70% at the lowest for the above cases, but I suspect that 50% may also work if necessary. If you are unsure, it is recommended that you consult with your sequencing core/provider on which index pairs to use. If consultation is not possible, you can always follow the criteria for library pooling with 17-24 TruDrop libraries, in that order, and it should work every time. The downside to this option is that certain index primers will be expended at a significantly higher rate resulting in varying turnover rates for index pairs.

If Sequencing 1-8 TruDrop libraries alongside lower percentages of other illumina compatible libraries (70% of the total pool or less is from other illumina compatible libraries) then follow the bellow pooling guidelines:
Use UDI0001, then UDI0002, then UDI0003, then UDI0004, then UDI0005, then UDI0006, then UDI0007, then UDI0008,
Or
Use UDI0017, then UDI0018, then UDI0019, then UDI0020, then UDI0021, then UDI0022, then UDI0023, then UDI0024,

If Sequencing 9-16 TruDrop libraries alongside lower percentages of other illumina compatible libraries (70% of the total pool or less is from other illumina compatible libraries) then follow the bellow pooling guidelines:
Use UDI0001, then UDI0002, then UDI0003, then UDI0004, then UDI0005, then UDI0006, then UDI0007, then UDI0008, then UDI0017, then UDI0018, then UDI0019, then UDI0020, then UDI0021, then UDI0022, then UDI0023, then UDI0024,
Or
Use UDI0017, then UDI0018, then UDI0019, then UDI0020, then UDI0021, then UDI0022, then UDI0023, then UDI0024, then UDI0001, then UDI0002, then UDI0003, then UDI0004, then UDI0005, then UDI0006, then UDI0007, then UDI0008,

If Sequencing 17-24 TruDrop libraries alongside lower percentages of other illumina compatible libraries (70% of the total pool or less is from other illumina compatible libraries) then follow the bellow pooling guidelines:
Use UDI0001, then UDI0002, then UDI0003, then UDI0004, then UDI0005, then UDI0006, then UDI0007, then UDI0008, then UDI0017, then UDI0018, then UDI0019, then UDI0020, then UDI0021, then UDI0022, then UDI0023, then UDI0024, then UDI0010, then UDI0011, then UDI0012, then UDI0013, then UDI0014, then UDI0015, then UDI0016, then UDI0025
Or
Use UDI0017, then UDI0018, then UDI0019, then UDI0020, then UDI0021, then UDI0022, then UDI0023, then UDI0024, then UDI0001, then UDI0002, then UDI0003, then UDI0004, then UDI0005, then UDI0006, then UDI0007, then UDI0008, then UDI0010, then UDI0011, then UDI0012, then UDI0013, then UDI0014, then UDI0015, then UDI0016, then UDI0025

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131 At this point the libraries should be treated like normal TruSeq libraries. Use normal TruSeq sequencing primers.

Libraries have been succesfully sequenced on NovaSeq with the following settings:
Read type: Paired- End
Read 1: 50 cycles or more (Cel Barcodes + UMI)
Read 2: 50 cycles or more (we normally use at least 100 cycles for transcript)
i7 index read: 8 cycles
i5 index read: 8 cycles

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