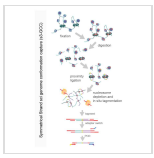

May 07, 2021

s3-GCC

Forked from s3-WGS

Andrew Adey[1], Ryan Mulqueen[1]

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1 Works for me dx.doi.org/10.17504/protocols.io.beb4jaqw

Combinatorial Indexing Protocols

Andrew Adey
Oregon Health & Science University

ABSTRACT

s3GCC (symmetrical strand single-cell combinatorial indexing; genome conformation capture) protocol for the capture of Hi-C like chimeric reads, generated through proximity ligation, as well as standard genomic reads.

See https://mulqueenr.github.io/schic/ for more information.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://www.biorxiv.org/content/10.1101/2021.01.11.425995v1

DOI

dx.doi.org/10.17504/protocols.io.beb4jaqw

PROTOCOL CITATION

Andrew Adey, Ryan Mulqueen 2021. s3-GCC. **protocols.io** https://dx.doi.org/10.17504/protocols.io.beb4jaqw

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

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FORK NOTE

FORK FROM

Forked from s3-WGS, Andrew Adey

KEYWORDS

single-cell, sci, s3, chromatin, sequencing, hi-c, hic

CREATED

Mar 29, 2020

LAST MODIFIED

May 07, 2021

PROTOCOL INTEGER ID

34908

MATERIALS TEXT

MATERIALS

CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #B7204S**

AluI - 5,000 units **New England Biolabs Catalog #R0137L**

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #M0202S**

Magnesium Chloride **Fisher Scientific Catalog #AC223210010**

IGEPAL-CA630 **Sigma Aldrich Catalog #I3021 SIGMA-ALDRICH**

DTT **Sigma Aldrich Catalog #D0632**

Tween-20 **Sigma-aldrich Catalog #P-7949**

Sodium Chloride **Fisher Scientific Catalog #S271-3**

4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) **Thermo Fisher Scientific Catalog #D1306**

Cell strainer, 35 µm **Corning Catalog #352235**

HEPES **Fisher Scientific Catalog #BP310-500**

Triton X-100 **Sigma Catalog #93426**

Tris-HCl **Life Technologies Catalog #AM9855**

High Sensitivity D5000 ScreenTape **Agilent Technologies Catalog #5067-5592**

High Sensitivity D5000 Reagents **Agilent Technologies Catalog #5067-5593**

Potassium hydroxide **Sigma Aldrich Catalog #1050121000**

UltraPure Distilled Water **Thermo Fisher Scientific Catalog #10977015**

Ethanol (EtOH) 200 Proof **Decon-Laboratories Inc Catalog #2701**

QIAquick PCR Purification Kit **Qiagen Catalog #28106**

Tagment DNA (TD) buffer **Illumina, Inc. Catalog #20018705**

NPM **Illumina, Inc. Catalog #n/a**

Pierce™ 16% Formaldehyde (w/v), Methanol-free **Thermo Fisher Catalog #28906**

Pierce™ Protease Inhibitor Mini Tablets, EDTA-free **Thermo Fisher Catalog #A32955**

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

NEBNext Q5U Master Mix – 50 rxns **New England Biolabs Catalog #M0597S**

Mag-Bind® TotalPure NGS **Omega Biotek Catalog #M1378-01**

Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES Membrane **Fisher Scientific Catalog #09-741-05**

Sodium dodecyl sulfate **Sigma Catalog #436143-25G**

Generation and storage of stock solutions.

1. **1M Potassium Hydroxide (KOH):** Generate 15mL of a 1M solution (841.575mg) in 15mL of ddH2O. Store at room temperature (RT).
2. **1M Hydrogen Chloride (HCl):** Generate 15mL of a 1M solution (546.89mg) in 15mL of ddH2O. Store at room temperature (RT).
3. **1M HEPES-KOH, pH 7.2:** Generate 1L of 1M HEPES solution (238.3g) in 900mL of ddH2O. pH to 7.2 with 1M KOH and bring up to 1L. Filter with a 0.2um rapid-flow filter and store at RT, protected from light.
4. **1M Tris-HCl, pH 7.4:** Generate 1L of 1M Tris solution (121.14g) in 900mL of ddH2O. pH to 7.4 with 1M HCl and bring up to 1L. Filter with a 0.2um rapid-flow filter and store at RT.
5. **5M Sodium Chloride (NaCl):** Generate 1L of a 5M solution (292.2g) in 1L of ddH2O. Filter with a 0.2um rapid-flow filter and store at RT.
6. **1M Magnesium Chloride ($MgCl_2$):** Generate 1L of a 1M solution (95.211g) in 1L of ddH2O. Filter with a 0.2um rapid-flow filter and store at RT.
7. **10% Tween-20:** Generate 15mL of a 10% (v/v) solution (1.5mL) in 13.5mL of ddH2O. Aliquot into light-protective 1.5mL tubes and store sheilded from light at 4C.
8. **10% IGEPAL-630:** Generate 15mL of a 10% (v/v) solution (1.5mL of 100% IGEPAL-630) in 13.5mL of ddH2O. Store at RT, protected from light.
9. **10% Triton-X100:** Generate 15mL of a 10% (v/v) solution (1.5mL of 100% Triton-X100) in 13.5mL of ddH2O. Store at RT.
10. **0.1% Sodium dodecyl-sulfate (SDS):** Generate 50mL of a 0.1% (w/v) solution (500mg SDS) in 50mL of ddH2O. Store at RT.
11. **5mg/mL 4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI):** Resuspend solid DAPI to 5 mg/mL in ddH20. Aliquot into light-protected tubes and store at -20C.
12. **0.1M DL-Dithiothreitol (DTT):** Generate 5mL of a 0.1M solution (77.125 mg) in 5mL of ddH2O. Store in aliquots at -20C.

Supplies List:

- 96-well PCR plates (Eppendorf, 951020427)
- 35 um cell strainer (VWR, 21008-948)

Instrument List:

- Table top centrifuge cooled to 4C with rotors for spinning 1) 96-well plates, and 2) 15 mL falcon tubes at 600 rcf
- Fluorescence Activated Cell Sorter (FACS), we use Sony SH800S
- Thermomixer with 96 well plate adapter (55C incubations at 300 rpm), we use Eppendorf Themomixer C
- Real-Time PCR instrument (Bio-Rad CFX Connect)
- DNA fluorometer or spectrophotometer (Qubit Fluorometer 2.0 is used in this protocol)
- Agilent Tapestation 4150 or Bioanalyzer
- NextSeq 500 using standard Mid 150 or High 150 protocol

BEFORE STARTING

Before starting, make sure to have stock solutions prepared (all of which are listed in the materials tab). Also ensure you have the appropriate instruments.

Also make sure you prepare the Tn5 with properly loading oligonucleotides as described in the first steps of the protocol.

Prepare oligoes and transposases

1 Attached is a s3 molecular layout per step. As well as oligonucleotides used in the protocol. Prior to starting, iTSM plate should be prepared following the protocols listed within this step.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

s3_oligos.xlsx

Tabs with oligonucleotides to be ordered and prepared:
- iTSM sci_WG
- A14_ME_LNA
- PCR_i5 primers
- PCR_i7 primers

2 **For generation of Tn5 enzyme.**

Generation and Purification of pTXB1.Tn5
by Andrew Adey,
Oregon Health & Science University
PREVIEW RUN

2.1 Generate LB agarose plates with **100 ug/mL Carbenicillin** following addgene suggested protocol.
https://www.addgene.org/mol-bio-reference/#antibiotics

2.2 Streak out E. coli stab with **pTXB1-Tn5** plasmid received from Addgene (https://www.addgene.org/60240/).

2.3 Select single colony with a sterile pipette tip and grow out colony in liquid LB with **100 ug/mL Carbenicillin**

2.4 Incubate with shaking (**250 rpm**) at **30 °C** overnight.

2.5 Extract plasmid using Qiagen Miniprep spin column following manufacturer's instructions.

2.6 Transform T7 Express LysY(NEB C3031) cells with extracted plasmid.

2.7 Grow out E. coli to generate long term storage stock in **25 % volume glycerol** in a cryovial tube as described in https://www.addgene.org/recipient-instructions/myplasmid/#long.
Store at **-80 °C** indefinitely.

2.8 Freshly streak out a LB agarose plates with **100 ug/mL Carbenicillin** from the glycerol stock. Allow to grow overnight at **30 °C** in an incubator.

2.9 Pick a single colony to inoculate **5 mL LB/carb** and incubate with shaking (**250 rpm**) at **30 °C** overnight.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

2.10 Add 2mL inoculum (expanded liquid colony) to **1 L LB/carb** in a 2 L capacity Erlenmeyer flask.

2.11 Grow to O.D.600 = .400-500 (**04:00:00 roughly**) at **30 °C** with shaking (**250 rpm**) , then let cool to **Room temperature** .

2.12 Add IPTG to **0.1 Milimolar (mM)** final concentration (**1 mL** of **100 Milimolar (mM)** stock) for induction at **18 °C** - **22 °C** on shaker with mild agitation (**20 rpm**) overnight.

We found no difference in temperature ranges from 18C to 22C during induction.

2.13 Spun down cells with a JA-10 rotor (Beckman Coulter) at **6000 x g** for **00:25:00** in 500 ml bottles (make sure bottles are balanced).

2.14 Decant supernatant and keep pellets on ice.

Safe stopping point: Pellets can be frozen in **-80 °C** and stored for a week.

2.15 Prepare **1 L** HEGX Buffer.

Reagent	Stock Concentration	Final Concentration	Amount of Stock
HEPES-KOH (pH 7.2)	1M	100mM	20mL
NaCl	Dry	800mM	46.8g
EDTA (pH 8.0)	0.5M	1mM	2mL
Glycerol	100%	10%	100mL
Triton-X100	100%	0.2%	2mL

2.16 **Perform steps 16 onwards in a cold room.**

Resuspend pellet in **75 mL** **4 °C (ice chilled)** HEGX Buffer and **3 tablets** of EDTA-free protease inhibitor

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

tablets in 100 ml beaker. Keep everything at **4 °C** .

2.17 Add a sterile magnetic stir bar and break up pellet on a stir plate in a cold room. Keep everything at **4 °C** .

2.18 Sonicate for **00:15:00** total:

Perform **00:00:15** **14 Watt pulses** pulses with a **00:00:15** break between each pulse. (30 pulses total).

Remove stir bar from beaker before sonicating. Keep everything on ice during sonication. Avoid foaming solution during sonication

2.19 Spin down lysate in 2 x 50 mL oak ridge tubes in JA-16 or JA-20 rotor.
Make sure tubes are balanced.

15000 rpm for **00:30:00** , **4 °C**

2.20 **Aliquot** **30 µl** **supernatant for future protein gel to check induction and purity.**

Pour remaining supernatant into clean 100 ml beaker with stir bar.

2.21 **Precipitate DNA:**

To supernatant in beaker, add **2 mL** dropwise of **5 % volume** Poly(ethyleneimine) Solution as it stirs on stir plate to precipitate DNA.

Reagent	Stock Concentration	Final Concentration	Amount of Stock
Poly(ethyleneimine) Solution	50%	5%	1mL
HEPES-KOH (pH 7.2)	1M	20mM	200 uL

Add PEI solution very slowly, dropwise down sides of beaker by a transfer pipette.
Solution will become cloudy.
Do not precipitate DNA too quickly.

2.22 Spin down supernatant in 2 x 50mL oak ridge tubes in JA-16 or JA-20 rotor.
Make sure tubes are balanced.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

12000 rpm for **00:10:00** , **4 °C**

2.23 **Aliquot 30 µl supernatant for future protein gel to check induction and purity.**

Pour remaining supernatant (henceforth called Tn5 lysate) into clean 500 ml beaker on ice.
Bring volume up to **150 mL** ice cold HEGX Buffer.

2.24 **Chitin Column preparation and loading Tn5 lysate:**

Clamp column on a stand in cold room for gravity flow.

Column type: Kimble-Chase Flex-Column Economy Columns with two 3-way valve stop-cocks

We've been using a 30cm (1 cm ID) column which enables you to add 24 mls volume during elution.

2.25 Add **10 mL** of Chitin resin using 10 ml serological pipet to column and allow resin to settle and pack on bottom of column

2.26 Equilibrate resin with **200 mL** of HEGX by gravity flow. Discard flow through.

2.27 Load **150 mL** of Tn5 Lysate by gravity flow over column.

SAVE this flow through for future protein gel to check induction and purity.

2.28 Once all protein solution is loaded on column, wash column with **200 mL** HEGX by gravity flow. Discard any flow through.

2.29 **Elution:**

Allow all wash buffer to drain through column and close bottom stopcock.

2.29.1 Add **24 mL** of HEGX with **[M]100 Milimolar (mM)** DTT directly to top of column material (Chitin) by a 25 ml serological pipette.

Reagent	Stock Concentration	Final Concentration	Amount of Stock
HEGX Buffer	1X	1X	24mL
DTT	Dry	100mM	0.37g

2.29.2 Open bottom stopcock and let **11 mL** of HEGX with **[M]100 Milimolar (mM)** DTT to flow through column. Measure volume by allowing volume to flow into 15mL falcon tube.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

2.29.3 Then close stop-cock and cap stop-cock to prevent any liquid from leaking from column. Cap stopcock on top of column too.

2.29.4 Leave HEGX/DTT solution on column in cold room for **48:00:00** to **72:00:00**

2.30 **Elution continued:**

Collect **9 mL** of flow through from column.

2.31 Dialyze against **1 L** of Dialysis buffer for **02:00:00** to **03:00:00** in a cold room.4

Reagent	Stock Concentration	Final Concentration	Amount of Stock
HEPES-KOH (pH 7.2)	1M	100mM	100mL
NaCl	5M	200mM	40mL
EDTA (pH 8.0)	0.5M	0.2mM	400uL
Glycerol	100%	20%	200mL
Triton-X100	100%	0.2%	2mL
Prior to adding DTT, Dialysis buffer can be stored at 4C			
DTT	Dry	2mM	0.308g

We use a Slide-a-Lyzer G2 cassettes with 10 KDa cutoff (ThermoFisher, No. 87731).

2.32 Exchange buffer with **1 L** of fresh dialysis buffer and dialyze overnight.

2.33 Measure O.D.280. It should be about 4.0.

2.34 **Aliquot 30 µl supernatant for future protein gel to check induction and purity.**

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

Run an SDS-PAGE gel following manufacturer's instruction on aliquots from steps 20, 23, 27 and 34 to check purity, and induction.

2.35 After dialysis, add approximately **6 mL** of sterile 100% glycerol to bring final concentration of glycerol to 55% (for approx. 15 ml final volume).

Aliquot in cryotubes and freeze at **-20 °C** . Protein is good for at least 1 year with proper storage.

3 **For loading Tn5 enzyme**.

- Load only a single oligonucleotide + mosaic end reverse compliment oligonucleotide per well.
- Load to a final concentration of equimolar 2.5uM oligonucleotide:mosaic end reverse compliment with 2.5uM Tn5.

Loading Tn5 Enzyme with sci-protocol Oligonucleotides
by Andrew Adey,
Oregon Health & Science University
PREVIEW RUN

3.1 Prepare **50 mL 2.125X Tn5 Dilution buffer** for protein dilution.

Reagent	Stock Concentration	Final Concentration	Amount of Stock
HEPES-KOH (pH 7.2)	1M	100mM	5mL
NaCl	5M	200mM	2mL
Glycerol	100%	25%	12.5mL
Triton-X100	100%	0.2%	100uL
ddH2O			30.4mL (to 50mL)
DTT	Dry	2mM	15.4 mg

Tn5 Dilution buffer can be stored at 4C for up to 2 months.

3.2 Prepare Mosaic End reverse compliment (ME'), i7, i5 oligonucleotides at
[M]100 Micromolar (µM) Tris-HCl buffer (pH 8.0)

See attached spreadsheet for oligonucleotide sequences.

Three sets of oligonucleotides are listed for both i5 and i7 Tn5 loading.
This yields (3 i5 sets) x (3 i7 sets)=9 uniquely identifiable 96 well plates or 864 unique well barcode combinations.

Mosaic End oligonucleotide sequence used for Tn5 loading is also listed within the spreadsheet.

Example_sciTn5_Oligos.xlsx

Synthesis quality of these oligonucleotides is critical. HPLC purification is essential. We find that Eurofins oligos

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

outperform IDT by roughly 10 fold in library complexity.

All indexes are designed to be 2 or greater Hamming distance from all others to allow for sequencing errors.

3.3 Preparation of dsDNA through annealing.

Volumes are adjusted for a single 96-well plate loading.

1. For each i5 barcoded oligo prepare the following reaction (8 total):

12.5 uL	100 uM i5 Tn5 Indexed oligo
12.5 uL	100 uM Mosaic End Reverse Compliment oligo
53.125 uL	2.125x Tn5 Dilution Buffer

Henceforth refered to as **i5/ME'**

2. For each i7 barcoded oligo prepare the following reaction (12 total):

8.5 uL	100 uM i7 Tn5 Indexed oligo
8.5 uL	100 uM Mosaic End Reverse Compliment oligo
36.125 uL	2.125x Tn5 Dilution Buffer

Henceforth refered to as **i7/ME'**

3.4 Anneal Oligo mixtures within a Thermocycler with the following reaction.

- **95 °C** **00:05:00**
- Slow ramp down to **20 °C at a rate of -2.5C/min**
- **20 °C hold**

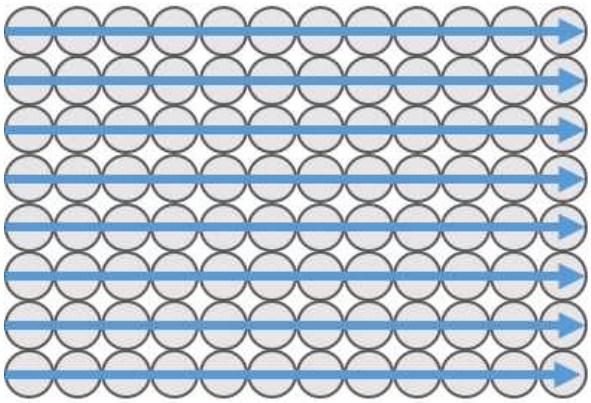
This results in **16 Micromolar (µM)** annealed oligo species per reaction (**i7/ME'** and i5/ME').

Oligoes should be freshly annealed prior to loading Tn5 transposome

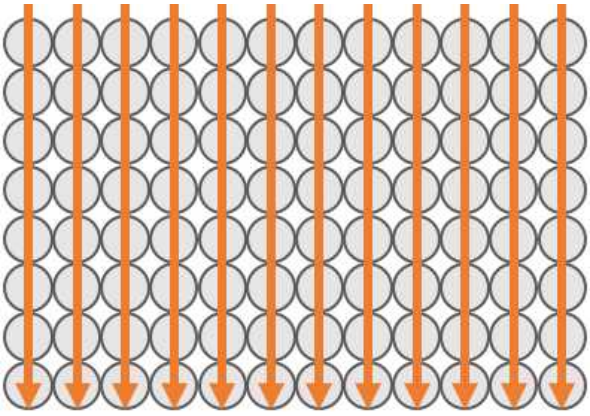
Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

3.5 **Prepare a 96-well plate with the following loading schema.**

1. Add **5 µl** of i5/ME' (**16 Micromolar (µM)**) to each respective wells in a row-wise fashion.



2. Add **5 µl** of i7/ME' (**16 Micromolar (µM)**) to each respective wells in a column-wise fasion.



This results in **10 µl i5/ME' and i7/ME' Indexed Oligos** at **8 Micromolar (µM)** /well

3.6 Prepare Tn5 protein as described in *"Generation and Purification of pTXB1.Tn5"* protocol.

Prior to loading Tn5 protein adjust NaCl concentration.
Combine:

1152 uL	Prepared Tn5
144 uL	5M NaCl

This adjusts salt to a final concentration of **555.55 Milimolar (mM)** NaCl

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

3.7 Add **12 µl** of salt-corrected Tn5 to each well of the 96 well plate.

Assemble the Tn5/oligo mixture via incubation at **25 °C** for **01:00:00** .

Store at -20C for no more than 8 months.

Prepare Buffers

4 Construct 50mL **Nuclei Isolation Buffer** (NIB-Tris):

Final Concentration	Stock Concentration	Volume of Stock
10 mM Tris HCl, pH 7.4	1M Tris-HCl, pH7.4	500 uL
10 mM NaCl	5M NaCl	100 uL
3mM MgCl2	1M MgCl2	150 uL
0.1 % Igepal	10% Igepal	500 uL
0.1 % Tween	10% Tween	500 uL
ddH20		to 50mL (add 48.25mL)

OPTIONAL: To prevent protease degradation, we also add 2 tables of Pierce Preotease Inhibitor Tablets, EDTA-Free to NIB following construction. We then vortex to fully dissolve tablets.

NIB is stable at **4 °C** for at least 1 month without noticable degradation in library quality or nuclei dissociation ability.

Store NIB on ice throughout nuclei dissociation and preparation of tagmentation plates.

5 Construct 50mL **Nuclei Isolation Buffer** (NIB-HEPES):

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

Final Concentration	Stock Concentration	Volume of Stock
10 mM HEPES-KOH, pH 7.2	1M HEPES-KOH, pH 7.2	500 uL
10 mM NaCl	5M NaCl	100 uL
3mM MgCl2	1M MgCl2	150 uL
0.1 % Igepal	10% Igepal	500 uL
0.1 % Tween	10% Tween	500 uL
ddH20		to 50mL (add 48.25mL)

OPTIONAL: To prevent protease degradation, we also add 2 tables of Pierce Preotease Inhibitor Tablets, EDTA-Free to NIB following construction. We then vortex to fully dissolve tablets.

NIB is stable at **4 °C** for at least 1 month without noticable degradation in library quality or nuclei dissociation ability.

Store NIB on ice throughout nuclei dissociation and preparation of tagmentation plates.

6 Construct 5mL of **10X NEBuffer 2.1**

Concentration (10X)	Stock Concentration	Volume of Stock
500mM NaCl	5M NaCl	500 uL
100mM Tris-HCl	1M Tris HCl (pH 7.9)	500 uL
100mM MgCl2	1M MgCl2	500 uL
1ng/uL BSA	100ng/uL BSA	50 uL
pH 7.9 @ 25C	H2O	3450 uL

Buffer can be stored at -20C for several months.

- **CRITICAL**: Ensure that this buffer is diluted to 1X before use.

Dissociate Adherent Cells

7 Different isolation techniques are h ighlighted across the s3 protocol family and can be used interchangeably for downstream processing. For s3WGS we primarliy used adherent cultured cell lines.

Note
Isolation of nuclei is dependent on the sample being used. And optimization should be performed. Below is an example of a nuclei isolation protocol to act as general use for primary brain tissue samples. Other tissues should follow a similar dounce homogenization protocol.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

7.1 Plate 1 million cells in T25 flask the day prior and harvest while cells are subconfluent.

7.2 **Wash adherent cell lines**

- Aspirate off media from flask
- Washed cells in flask with **5 mL ice cold 1X PBS** , and aspirate off

7.3 **Trypsinize cells:**

- Add **5 mL TrypLE** to flask an incubate at **37 °C** for **00:15:00**
- Collect cells and move to 15mL tube on ice.

7.4 Spin down nuclei solution at **400 x g, 00:10:00** at **4 °C** .

7.5 Resuspend pellet in **1 mL NIB:HEPES**.

Let nuclei incubate for **00:10:00** **On ice**

7.6 Repeat the spin down and resuspension (Repeat step 6.4-6.5) for a second wash.

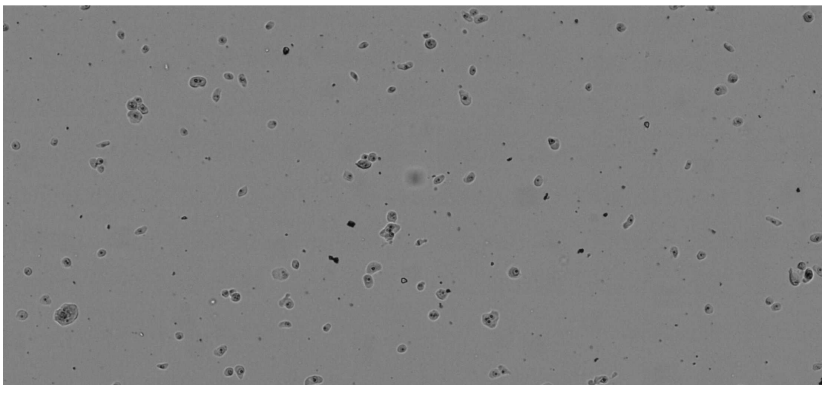
7.7 Resuspend cells in **300 µl NIB:HEPES** and hold on ice.

Quantify Nuclei Concentration

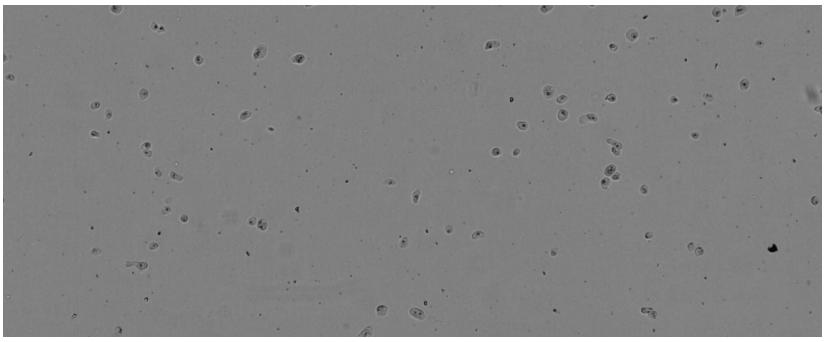
8 Take aliquot of sample and dilute (10uL of nuclei suspension in **90 µl NIB:HEPES** for a 1:10 dilution).

9 Determine nuclei/uL concentration of diluted aliquot through Hemocytometer or BioRad TC20 Automated Cell Counter using manufacturer's recommended protocols.

Adjust calculated concentration for the stock nuclei suspension.



Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw



Example TC20 image of isolated nuclei.

10 Separate nuclei into 1 million nuclei aliquots and transfer into 5mL tubes.
Add volume of **NIB:HEPES** to suspend 1M nuclei aliquots in **5 mL** .

We generally generate multiple replicates of the 1M nuclei aliquots. We have also done up to 3M nuclei aliquots without any change to protocol which performed the same.

Nuclei Fixation

11 To the 5mL nuclei suspension, add **246 µl 16% formaldehyde** from a freshly opened ampule.
- 0.75% formaldehyde final concentration is ideal.

12 Incubate nuclei at **Room temperature** for **00:10:00** on an orbital shaker set to **50 rpm** .

13 Centrifuge nuclei at **500 x g, 4°C, 00:05:00** to pellet nuclei. Aspirate off solution, being careful not to disturb the pellet.

14 Wash pellet with **1 mL NIB:Tris**.
- Tris will quench the formaldehyde

Centrifuge nuclei at **500 x g, 4°C, 00:05:00** to pellet nuclei. Aspirate off solution, being careful not to disturb the pellet.

15 Wash pellet with **200 µl 1X NEBuffer 2.1 (ice cold)**
- **CRITICAL:** Buffer recipe listed above is 10X. Ensure you dilute to 1X before use.

Transfer resuspended nucleito 1.5mL tube for improved pelleting.

Centrifuge nuclei at **500 x g, 4°C, 00:05:00** to pellet nuclei. Aspirate off solution, being careful not to disturb the pellet.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

Nucleosome Depletion

16 Resuspend pellet in **760 µl** **1X NEBuffer 2.1**

17 Add **40 µl** of **1% SDS** and incubate at **300 rpm, 37°C, 00:20:00** on a ThermoMixer

- 0.05% final concentration SDS is ideal

18 Centrifuge nuclei at **500 x g, 4°C, 00:05:00** to pellet nuclei. Aspirate off solution, being careful not to disturb the pellet.

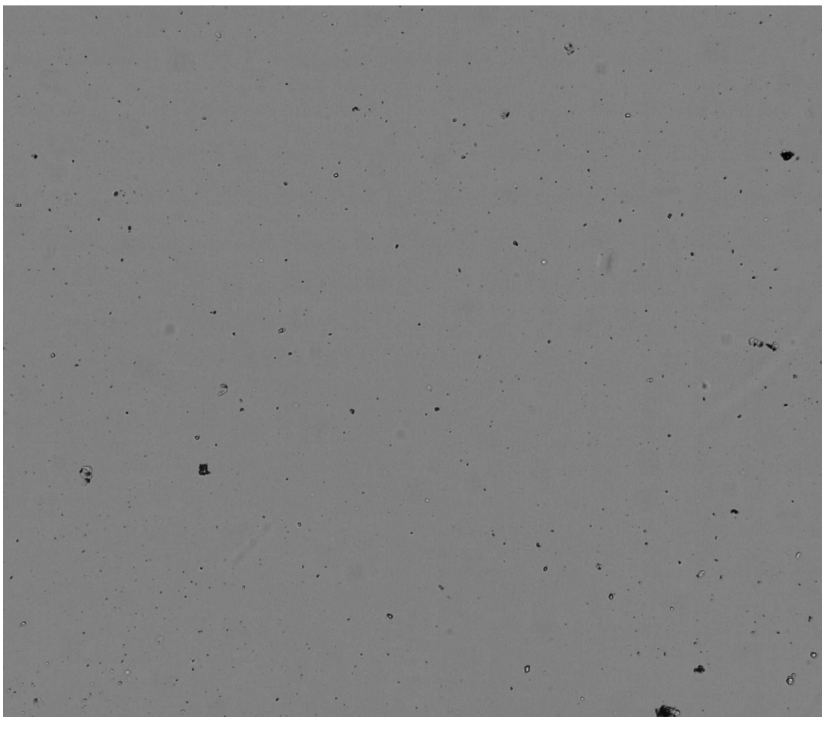
19 Resuspend pellet in **50 µl** **NIB:Tris.** If there are multiple replicates they can be pooled at this point.

If there is significant clumping, nuclei can bed loosed by a 10 min incubation in **NIB:Tris** and then triturated. If this does not work, loose strokes (B) on a douce homogenizer works as well.

20 Take aliquot of sample and dilute (5uL of nuclei suspension in **45 µl** **NIB:Tris** for a 1:10 dilution).

21 Determine nuclei/uL concentration of diluted aliquot through Hemocytometer or BioRad TC20 Automated Cell Counter using manufacturer's recommended protocols.

Adjust calculated concentration for the stock nuclei suspension.



Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

Example of fixed and nucleosome depleted nuclei imaged on BioRad TC20. Note ensure that nuclei count accounts for the debris present and an accurate nuclear count is attained.

Restriction Digest and Proximity Ligation

22 **For restriction digest, use a promiscuous cutter. We have used AluI which is a blunt end 4-cutter.**

Combined pooled nuclei into a single tube.

23 Centrifuge nuclei at **500 x g, 4°C, 00:05:00** to pellet nuclei. Aspirate off solution, being careful not to disturb the pellet.

24 Resuspend pellet in **90 µl 1X Cutsmart Buffer**

25 Add **10 µl 10 U/uL AluI Enzyme**

26 Incubate at **300 rpm, 37°C, 02:00:00** on a ThermoMixer

27 Centrifuge nuclei at **500 x g, 4°C, 00:05:00** to pellet nuclei. Aspirate off solution, being careful not to disturb the pellet.

28 **For proximity ligation**
Resuspend nuclei in 100uL of the following mixture:

Reagent	Volume for 100uL mixture
10X T4 DNA Ligase Buffer + ATP	10 uL
ddH2O	88 uL
0.1 % TritonX-100	1 uL (final concentration 0.01%)
0.1 M DTT	0.5 uL (final concentration 0.5mM)
T4 DNA (400 U/uL)	0.5 uL (200 U total)

29 Incubate reaction at **16 °C** **Overnight (12-16 hours)**

30 Centrifuge nuclei at **500 x g, 4°C, 00:05:00** to pellet nuclei. Aspirate off solution, being careful not to disturb the

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

pellet.

31 Resuspend pellet in **200 µl NIB:HEPES**

32 Take aliquot of sample and dilute (5uL of nuclei suspension in **45 µl NIB:HEPES** for a 1:10 dilution).

33 Determine nuclei/uL concentration of diluted aliquot through Hemocytometer or BioRad TC20 Automated Cell Counter using manufacturer's recommended protocols.

Adjust calculated concentration for the stock nuclei suspension.

34 Dilute nuclei to 500 nuclei/uL with NIB:HEPES.

Tagmentation

35 **Prepare tagmentation plate**

1. Combine **420 µl 500 nuc/uL solution** with **540 µl 2X TD Buffer** per 96 well iTSM plate.
2. Pipette out 8uL per well of TD Buffer: Nuclei solution.
3. Add 1uL of 2.5uM uniquely indexed SBS12-U-ME TSM per well.

Final reagents per well in tagmentation plate.

Component	Volume per well (9uL total)
~20ng DNA	3.5 ul (~1750 nuclei at a concentration of 500 nuclei/uL)
iTSM	1 uL 2.5uM SBS12-U-ME TSM
TD Buffer	4.5ul 2X TD Buffer

2X TD Buffer available from Nextera XT Kit

Hold SBS12-U-ME TSM plate on ice while pipetting out. Also hold nuclei and tagmentation plate on ice until plate is fully prepared.

36 Incubate at **55 °C** for **00:10:00** while shaking at **300 rpm** .

Shaking helps keep nuclei suspended during tagmentation.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

37 Place plate on ice following incubation and hold for **00:02:00** to allow reactions to drop temperature.

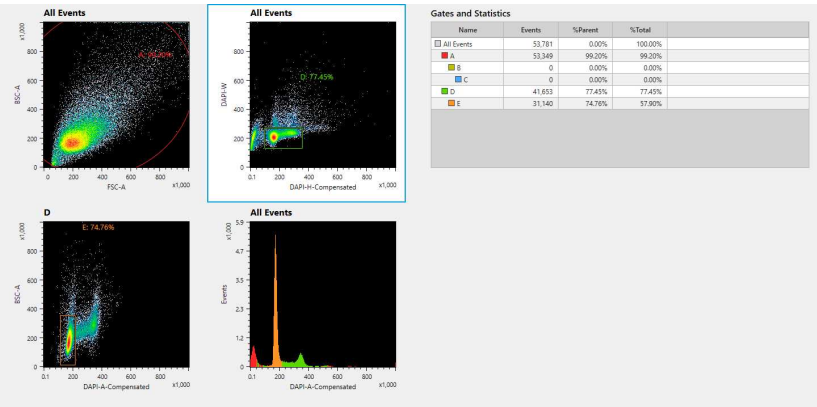
38 Pool the full plate while maintaining plate and pool on ice. Add **2 µl 5mg/mL DAPI** to pool. Hold pool on ice while preparing the PCR plate. Nuclei are stable on ice for several hours.

If there are any visible chunks in pool, strain again through 40um cell strainer.

Single-cell sorting

39 Prepare a 96 well plate with **9 µl 1X TD Buffer** per well , diluted with ddH2O.

40



Example gating strategy on DAPI+ nuclei using the Sony SH800S Cell Sorter.

From the DAPI stained pool, sort 15 nuclei per well mimicking the above nuclei gating strategy. If possible, hold the sample chamber and pool at **4 °C** in your sorter settings.

The number of nuclei per well for PCR is determined by the number of uniquely indexed transposases going into tagmentation. For 96 reactions (a single iTSM plate), it has been emperically deteremined to be 15 nuclei per well based on a 5% barnyard collision rate.

41 Following sorting into the 96 well plate, centrifuge the plate at **500 x g, 4°C, 00:05:00** to ensure nuclei are within the solution.

- **SAFE STOPPING POINT:** Plates can be frozen (stored at **-20 °C**)after spin down for at least 1 week without measurable decrease in library quality.

Gap fill and adapter switching

42 Add **1 µl 0.1% SDS** to each well.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

43 Add **4 µl NPM** to each well.

It is crucial to dd 0.1% SDS and NPM in step-wise fashion and not pre-combined. High SDS concentration will disrupt polymerases in NPM.

44 **Gap-fill** molecules by incubating plate at **72 °C** for **00:10:00** .

- After extension, hold plate **On ice** to slow reaction.

45 Add **1.5 µl A14-LNA_ME at 1uM** to a final concentration of **100 Nanomolar (nM)**

46 Use multiple rounds of linear extension to switch adapter on one side of the molecules.

1. **98 °C** for **00:00:30**
2. **98 °C** for **00:00:10**
3. **59 °C** for **00:00:20**
4. **72 °C** for **00:00:10**
5. Go to step 2 9 times for 10 total cycles.
6. Hold at **10 °C**

47 Add **1 µl 1% (v/v) Triton X-100** .

- It is important that the ratio is 10:1 TritonX:SDS to quench SDS prior to PCR polymerase use.

SAFE STOPPING POINT: Plates can be frozen (stored at **-20 °C**)after spin down for at least 10 weeks without measurable decrease in library quality.

PCR

48 Combine the following reagents for a RT-PCR reaction directly to the 96 well plate contained the samples. ddH2O, Q5U Master Mix and 100X SYBR can be made as a mastermix prior to added to wells.

Reagent	**Volume per well**
Sample	16.5 ul
PCR primers: S70X (TruSeq i7) 10uM	2.5 uL
PCR primers: N50X (Nextera i5) 10uM	2.5 uL
ddH2O	3 uL
Q5U 2X Master Mix	25 ul
100X SYBR	0.5uL
Total	**50 ul for PCR**

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

49 Perform a real-time PCR , measuring SYBR fluorecence every cycle.

1. **98 °C** for **00:00:30**
2. **98 °C** for **00:00:10**
3. **55 °C** for **00:00:20**
4. **72 °C** for **00:00:30**
5. Measure SYBR fluoresence.
6. **72 °C** for **00:00:10**
7. Go to step 2 16 more times for 17 cycles total.
8. Hold at **72 °C** for **00:02:00**
9. Hold at **10 °C**

PCR takes between 15-17 cycles. Use SYBR fluoresence to guide when to pull libraries. Once fluoresence stops exponential growth, the libraries are ready to hold at 72C and then remove.

SAFE STOPPING POINT: Plates can be frozen (stored at **-20 °C**)after spin down for at least 10 weeks without measurable decrease in library quality. Can also be held at **4 °C** for at least 5 weeks.

Library clean up

50 **Pool post-PCR Product**

Pool 25 uL from each well into 15mL conical tube.

51 **Concentrate DNA via column clean up**

Run full pool volume through Qiaquick PCR purification column following manufacturer's protocol.

Qiagen QIAquick PCR Purification Kit Protocol
by Michael Crone
PREVIEW RUN

Elute in **50 µl 10 mM Tris-HCl pH 8.0** .

51.1 Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. It is not necessary to remove mineral oil or kerosene.

Buffer PB **Contributed by users**

51.2 If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

10 µl

Sodium acetate **P212121**

51.3 Place a QIAquick spin column in a provided 2 ml collection tube.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

51.4 To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s. Discard flow-through. Place the QIAquick column back into the same tube.

00:01:00

51.5 To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.

00:01:00

Buffer PE **Contributed by users**

51.6 Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

00:01:00

51.7 Place QIAquick column in a clean 1.5 ml microcentrifuge tube.

51.8 To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

51.9 If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

52 Clean by size selection with SPRI beads

Perform a 1X SPRI bead size selection (selecting for DNA > 200 bp).

52.1 Add **50 µl MagPure NGS Binding Beads** to column elution, once beads are at room temperature.

- Ensure beads are fully mixed prior to taking from stock. Vortex for at least 10 seconds immediately before pipetting out.

Let mixture incubate at room temperature for **00:05:00** .

52.2 Place tube on magnetic rack and wait for magnetic beads to pellet and elution to fully clear (roughly **00:02:00**).

Remove full volume of elution without disrupting bead pellet. Discard elution.

52.3 Resuspend bead pellet in freshly prepared **100 µl 80% ethanol (v/v)** .

Remove full volume of elution without disrupting bead pellet. Discard ethanol.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

52.4 Resuspend bead pellet in freshly prepared **100 µl 80% ethanol (v/v)** .

Remove full volume of elution without disrupting bead pellet. Discard ethanol.

52.5 Remove tube from magnetic rack and spin briefly on tapletop centrifuge.

Return to magnetic rack and once beads are pelleted again, remove any remaining ethanol from bottom of tube.

52.6 Resuspend beads off magnetic rack in **31 µl 10 mM Tris-HCl pH 8.0** .

Let mixture incubate at room temperature for **00:05:00** for DNA to fully become suspended.

Place tube on magnetic rack and wait for magnetic beads to pellet and elution to fully clear (roughly **00:02:00**).

Remove full volume of elution without disrupting bead pellet and move elution to clean tube.

53 Qubit DNA HS Quantification

Quantify DNA concentration with 1uL eluted sample on Qubit DNA High-sensitivity kit following manufacturer's protocol.

Qubit dsDNA HS/BR Assay
by Sarah Hessen-Schmidt,
University of Southern California

PREVIEW RUN

53.1 Label small Qubit tube lids for the number of standards and samples. Qubit requires 2 standards.

53.2 Make Qubit working solution by diluting Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS buffer in a large Qubit tube. The final volume in each tube must be 200µl.
(number of samples + 3 for control and standards) x 200µl

53.3 Prepare 2 standard tubes. Add 190µl of working solution to each standard tube. Add 10µl of each standard to the appropriate tube and mix by vortexing 2-3 seconds and quick spin down.

53.4 Prepare sample and control tubes by adding 199µl of working solution to each tube and 1µl of sample to the appropriate tube. Mix by vortexing 2-3 seconds and quick spin down. Alternate volumes of sample (1-20µl) and working solution (180-199µl) may be added to reach a total volume of 200µl.

53.5 Allow all tubes to incubate at room temperature for 2 minutes in a dark drawer
00:02:00

53.6 On Qubit fluorometer home screen select assay type (Quant-iT dsDNA, BR or HS)

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

53.7 On the Standards Screen press Yes to run a new calibration. Insert Standard #1 tube, close lid and press read. Remove Standard #1 and insert Standard #2 tube. Close lid and press read. Remove Standard #2.

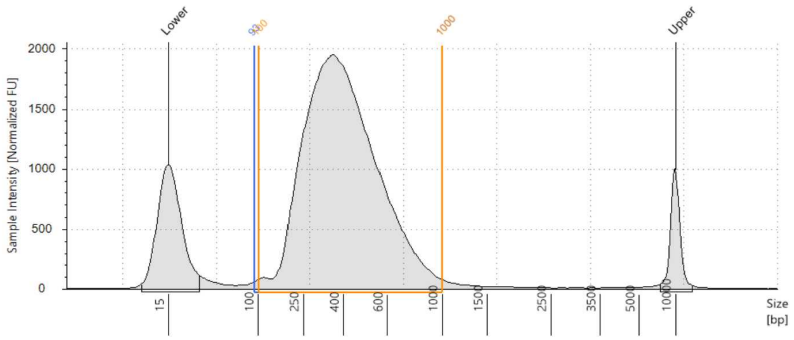
53.8 Once calibration has been completed the Sample Screen will be displayed. Insert a sample tube in to the fluorometer, close the lid and press Read. The value displayed is the concentration after you sample was diluted into the assay tube. To calculate the concentration of the original sample use the Qubit Dilution Calculator by pressing Calculate Stock Conc. Select the volume of you original sample that you added to the tube (1-20µl) and the fluorometer will calculate for you.

53.9 To save the data from your calculation press Save on the Dilution Calculator Screen and the last value will be save as a .CSV file tagged with a time and date stamp.

53.10 Remove the sample tube, insert the next sample tube and close the lid. Press Read Next Sample. Repeat until all sample tube values have been recorded.

54 Agilent Tapestation 4150 Quantification

Dilute sample to 2 ng/uL based on read out of Qubit by addition of 10mM Tris-HCl pH 8.0.
Run 2 uL sample on **Agilent Tapestation 4150 DNA High Sensitivity D5000 or D1000** following manufacturer's protocol.



Example **Agilent Tapestation 4150 DNA High Sensitivity D5000** trace of a s3GCC library.

Sequencing

55 Use Tapestation software to quantify DNA from 200-1000bp size.
Dilute library to 1nM and prepare for sequencing following Illumina protocols.

56 Load libraries according to standard loading concentrations suggested by Illumina with the following cycle counts per read. Sequencing is standard chemistry.

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw

Read	Read 1	Read 2	Index 1	Index 2
Number of Cycles	74	74	10	10
Custom Index Primer?	No	No	No	No

Citation: Andrew Adey, Ryan Mulqueen (05/07/2021). s3-GCC. https://dx.doi.org/10.17504/protocols.io.beb4jaqw