In December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic agent of coronavirus disease 2019 (COVID-19), emerged in Wuhan, China. Since then, it has rapidly spread worldwide (1–3), causing 7,039,918 confirmed cases, including 404,396 deaths, in 188 countries or regions as of June 9, 2020 (4). Because SARS-CoV-2 has shown the capacity to spread rapidly and lead to a range of manifestations in infected persons, from asymptomatic infection to mild, severe, or fatal disease, it is essential to identify genetic variants to track spread and understand any changes in transmissibility, tropism, and pathogenesis.

We describe the design and use of 2 PCR-based methods for sequencing SARS-CoV-2 clinical specimens. The first is a multiplex PCR panel, followed by sequencing on either the Oxford Nanopore MinION apparatus (https://nanoporetech.com) or an Illumina MiSeq apparatus (https://www.illumina.com). When coupled with MinION sequencing, our protocol can be implemented outside a traditional laboratory and can be completed in a single workday, similar to previous mobile genomic surveillance of Ebola and Zika virus outbreaks (5,6). In addition, we provide a complementary singleplex, nested PCR strategy, which improves sensitivity for samples with lower viral load and is compatible with Sanger sequencing.

The Study
On January 10, 2020, the first SARS-CoV-2 genome sequence was released online (7). That day, we designed 2 complementary panels of primers to amplify the virus genome for sequencing.

For the first panel, we used the PRIMAL primer design tool (5) to design multiplex PCRs to amplify the genome by using only a few PCRs (Appendix, https://wwwnc.cdc.gov/EID/article/26/10/20-1800-App1.pdf). The final design consists of 6 pools of primers optimized for sensitivity and assay flexibility. The amplicons average 550 bp with 100-bp overlaps to enable sequencing on either the Oxford MinION or Illumina MiSeq.

For the second panel, we designed sets of primers to generate nested, tiling amplicons across the SARS-CoV-2 genome (Appendix) for enhanced sensitivity in samples with lower viral loads. Each amplicon is 322–1,030 bp with an average overlap of 80 bp. These amplicons are designed to be amplified and sequenced individually on Sanger instruments but might also be pooled for sequencing on next-generation sequencing platforms.

To determine the sensitivity of each sequencing strategy, we generated a set of 6 ten-fold serial dilutions of a SARS-CoV-2 isolate (J. Harcourt, unpub. data, https://doi.org/10.1101/2020.03.02.972935). Virus RNA was diluted into a constant background of A549 human cell line total nucleic acid (RNaseP cycle threshold [Ct] 29). We quantitated each dilution by using the Centers for Disease Control and Prevention SARS-CoV-2 real-time reverse transcription PCR for the nucleocapsid 2 gene (8). The 6

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (C. Paden, Y. Tao, K. Queen, J. Zhang, Y. Li, A. Uehara, S. Tong); IHRC, Atlanta (J. Zhang); Oak Ridge Institute for Science and Education, Oak Ridge, Tennessee, USA (A. Uehara)

DOI: https://doi.org/10.3201/eid2610.20.1800

1These authors contributed equally to this article.
Figure 1. Limits of detection for sequencing severe acute respiratory syndrome coronavirus 2. Triplicate serial dilutions of virus isolate A12 (J. Harcourt, unpub. data, https://doi.org/10.1101/2020.03.02.972935) were amplified by using the singleplex or multiplex primer set. Multiplex amplicons were barcoded, library-prepped, and sequenced on an Oxford MinION apparatus (https://nanoporetech.com) or an Illumina MiSeq apparatus (https://www.illumina.com). A) Percentage of reads that map to the virus genome for each sample. B) Percentage of virus genome that is covered at >20× depth by the multiplex amplicons on the MinION (black) or >100× depth on the MiSeq (orange), or covered by the nested, singleplex amplicons (gray) (measured by presence or absence on a gel). C) Real-time analysis of MinION sequencing data. Each data point represents the average 20× genome coverage of three replicates. NTC, nontemplate controls (human cell nucleic acid carried through the PCR and library preparation). Asterisk (*) indicates that samples were not analyzed at that dilution.

dilutions spanned C_{t} values from 22 to 37, corresponding to ≈2 × 10^{2} to 1.8 × 10^{5} copies. We amplified triplicate samples at each dilution by using the multiplex PCR pools. Next, we pooled, barcoded, and made libraries from amplicons of each sample by using the ligation-based kit and PCR barcode expansion kit (Appendix). MinION sequencing was performed on an R9.4.1 or R10.3 flow cell (Oxford) until we obtained >1–2 million raw reads. From those reads, 50%–60% of them could be demultiplexed. In

| Virus titer (cycle threshold) | % Coverage, 20×† | Indels | Indel bases | Single-nucleotide polymorphisms | % Identity† |
|-----------------------------|-----------------|--------|-------------|---------------------------------|-------------|
| 22.3                        | 99.659          | 0      | 0           | 0                               | 100         |
|                             | 99.722          | 0      | 0           | 0                               | 100         |
|                             | 99.635          | 0      | 0           | 0                               | 100         |
| 25.7                        | 99.635          | 0      | 0           | 0                               | 100         |
|                             | 99.615          | 0      | 0           | 0                               | 100         |
|                             | 99.642          | 0      | 0           | 0                               | 100         |
| 29.2                        | 99.508          | 0      | 0           | 0                               | 100         |
|                             | 99.635          | 0      | 0           | 0                               | 100         |
|                             | 99.615          | 0      | 0           | 0                               | 100         |
| 33.2                        | 93.024          | 1      | 1           | 0                               | 100         |
|                             | 93.603          | 2      | 35          | 0                               | 100         |
|                             | 87.894          | 0      | 0           | 0                               | 100         |
| 35.6                        | 41.653          | 1      | 1           | 0                               | 100         |
|                             | 51.266          | 0      | 0           | 1                               | 99.993      |
|                             | 50.621          | 1      | 15          | 2                               | 99.987      |
| 37.6                        | 14.634          | 0      | 0           | 1                               | 99.977      |
|                             | 9.317           | 0      | 0           | 0                               | 100         |
|                             | 12.363          | 0      | 0           | 0                               | 100         |

*Because the 5′ and 3′ ends are primer sequences, 100% coverage is not possible.
†Percentage of covered bases identical to reference sequence, excludes indels and low-coverage bases.
addition, we sequenced these amplicons by using the Illumina MiSeq for comparison (Appendix).

For MinION sequencing, the reads were basecalled and analyzed by using an in-house read mapping pipeline (Appendix). For samples with $C_t \leq 29$, we obtained >99% SARS-CoV-2 reads and >99% genome coverage at 20× depth, decreasing to an average of 93% genome coverage at $C_t 33.2$ and 48% at $C_t 35$ (Figure 1, panels A, B). Furthermore, we were able to obtain full genomes at >20× reading depth within the first 40–60 min of sequencing (Figure 1, panel C).

Consensus accuracy, including single-nucleotide polymorphisms and indels, is critical for determining coronavirus lineage and transmission networks. For high-consensus–level accuracy, we filtered reads based on length, mapped them to the reference sequence (GenBank accession no. RefSeq NC_045512), trimmed primers based on position, and called variants with Medaka (https://github.com) (Appendix). Each Medaka variant was filtered by coverage depth (>20×) and by the Medaka model-derived variant quality (>30). We used the variant quality score as a heuristic to filter remaining noise from the Medaka variants compared with Sanger-derived sequences. After these steps, the data approaches 100% consensus accuracy (Table 1). Identical results were found by using the R9.4.1 pore through samples with $C_t$ values through 33.2. The larger deletions in some of the samples with $C_t$ values >33.2 (Table 1) do not appear to be sequencing errors because they are also detected as minor populations within higher-titer samples.

In the MiSeq data, we observed a similar trend in percent genome coverage at 100× depth, and a slightly lower percentage mapped reads compared with Nanopore data (Figure 1, panels A, B). Increased read depth using the MiSeq potentially enables increased sample throughput. However, the number of available unique dual indices limits actual throughput.

For the nested, singleplex PCR panel, we amplified the same serial dilutions with each nested primer set (Appendix). The endpoint dilution for full-genome coverage is a $C_t \approx 35$ (Figure 1, panel B). At the $C_t 37$ dilution, we observed major amplicon dropout; at this dilution, there are <10 copies of the genome on average/reaction.

These protocols enabled rapid sequencing of initial clinical cases of infection with SARS-CoV-2 in the United States. For these cases, we amplified the virus genome by using the singleplex PCR and sequenced the amplicons by using the MinION and Sanger instruments to validate MinION consensus accuracy. The MinION produced full-length genomes in <20 min of sequencing, and Sanger data was available the following day.

We used the multiplex PCR strategy for subsequent SARS-CoV-2 clinical cases ($n = 167$) with $C_t$ values ranging from 15.7 to 40 (mean 28.8, median
Table 2. Comparison of input, time, and cost requirements for sequencing 1 or 96 specimens of severe acute respiratory syndrome coronavirus 2

| Method                  | Input, µL* | Turnaround time | Approximate cost/sample† | Turnaround time | Approximate cost/sample† |
|-------------------------|------------|----------------|--------------------------|----------------|--------------------------|
| Multiplex/MinION        | 10         | 6–8 h          | $528.70                  | 8–10 h         | $35.88                   |
| Multiplex/MiSeq         | 10         | 30–68 h‡       | $1,443.29                | 30–68 h‡       | $57.87                   |
| Singleplex/Sanger       | 190        | 16–18 h        | $354.40                  | 17–19 d        | $354.40                  |

*Assumes a process with 200 µL of resuspended respiratory specimen (from a total of 2 mL), extracted, and eluted into 100 µL. See Appendix (https://wwwnc.cdc.gov/EID/article/26/10/20-1800-App1.pdf) for details.
†Includes specific enzyme and reagent costs; excludes common laboratory supplies and labor costs.
‡Varies according to the sequencing kit used.

In cases with a C<sub>v</sub> < 30, we observed an average of 99.02% specific reads and 99.2% genome coverage at >20× depth (Figure 2, panels A, B). Between C<sub>v</sub> 30 and 33, genome coverage varied by sample, and decreased dramatically at higher C<sub>v</sub> values, analogous to the isolate validation data. For these samples, we multiplexed 20–40 barcoded samples/flowcell. Enough data are obtained with 60 min of MinION sequencing for most samples, although for higher titer samples, 10–20 min of sequencing is sufficient (Figure 2, panel C). Up-to-date primer sequences, protocols, and analysis scripts are available on GitHub (https://github.com/CDCgov/SARS-CoV-2_Sequencing/tree/master/protocols/CDC-Comprehensive). Data from this study is deposited in the National Center for Biotechnology Information Sequence Read Archive (BioProjects PRJNA622817 and PRJNA610248).

Conclusions

Full-genome sequencing is a critical tool in understanding emerging viruses. Initial sequencing of SARS-CoV-2 showed limited genetic variation (9,10). However, some signature variants have been useful for describing the introduction and transmission dynamics of the virus (11; T. Bedford et al., unpub. data, https://doi.org/10.1101/2020.04.02.20051417; X. Deng et al., unpub. data, https://doi.org/10.1101/2020.03.27.20044925; M. Worobey et al., unpub. data, https://doi.org/10.1101/2020.05.21.109322).

We provide 2 validated PCR target-enrichment strategies that can be used with MinION, MiSeq, and Sanger platforms for sequencing SARS-CoV-2 clinical specimens. These strategies ensure that most laboratories have access to ≥1 strategies. The multiplex PCR strategy is effective at generating full genome sequences up to C<sub>v</sub> 33. The singleplex, nested PCR is effective up to C<sub>v</sub> 35, varying based on sample quality. The turnaround time for the multiplex PCR MinION protocol is ≈8 hours from nucleic acid to consensus sequence and that for Sanger sequencing is ≈14 18 hours (Table 2). The multiplex PCR protocols offer an efficient, cost-effective, scalable system, and add little time and complexity as sample numbers increase (Table 2). Results from this study suggest multiplex PCR might be used effectively for routine sequencing, complemented by singleplex, nested PCR for low-titer virus samples and confirmation sequencing.

Acknowledgments

We thank the Respiratory Viruses Branch, Division of Viral Disease, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, for helping in organizing samples used in this study.

About the Author

Dr. Paden is a virologist and bioinformatician in the Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA. His primary research interest is identifying and characterizing novel and emerging pathogens.

References

1. Holshue ML, DeBolt C, Lindquist S, Lofy KH, Wiesman J, Bruce H, et al.; Washington State 2019-nCoV Case Investigation Team. First case of 2019 novel coronavirus in the United States. N Engl J Med. 2020;382:929–36. https://doi.org/10.1056/NEJMoa2001191
2. Patel A, Jernigan DB, Abdirizak F, Abedi G, Aggarwal S, Albina D, et al.; 2019-nCoV CDC Response Team. Initial public health response and interim clinical guidance for the 2019 novel coronavirus outbreak—United States, December 31, 2019–February 4, 2020. MMWR Morb Mortal Wkly Rep. 2020;69:140–6. https://doi.org/10.15585/mmwr.mm6905e1
3. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health concern. Lancet. 2020;395:470–3. https://doi.org/10.1016/S0140-6736(20)30185-9
4. World Health Organization. Coronavirus disease 2019 (COVID-19) situation report 141 [cited 2020 Jun 9]. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports
5. Quick J, Grubaugh ND, Pullan ST, Claro IM, Smith AD, Gangavarapu K, et al. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nat Protoc. 2017;12:1261–76. https://doi.org/10.1038/nprot.2017.066
6. Quick J, Loman NJ, Duraffour S, Simpson JT, Severi E, Cowley L, et al. Real-time, portable genome sequencing for Ebola surveillance. Nature. 2016;530:228–32. https://doi.org/10.1038/nature16996

7. Holmes EC, Novel YZ. 2019 coronavirus genome, 2020 [cited 2020 Apr 5]. http://virological.org/t/novel-2019-coronavirus-genome/319

8. COVID-19 Investigation Team. Clinical and virologic characteristics of the first 12 patients with coronavirus disease 2019 (COVID-19) in the United States. Nat Med. 2020;26:861–8. https://doi.org/10.1038/s41591-020-0877-5

9. Andersen K. Clock and TMRCA based on 27 genomes, 2020 [cited 2020 Jan 25]. http://virological.org/t/clock-and-tmrca-based-on-27-genomes/347

10. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, et al. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet. 2020;395:565–74. https://doi.org/10.1016/S0140-6736(20)30251-8

11. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. The proximal origin of SARS-CoV-2. Nat Med. 2020;26:450–2. https://doi.org/10.1038/s41591-020-0820-9

Address for correspondence: Suxiang Tong, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop H18-6, Atlanta, GA 30329-4027, USA; email: sot1@cdc.gov

July 2020

Emerging Viruses

- Case Manifestations and Public Health Response for Outbreak of Meningococcal W Disease, Central Australia, 2017
- Transmission of Chikungunya Virus in an Urban Slum, Brazil
- Public Health Role of Academic Medical Center in Community Outbreak of Hepatitis A, San Diego County, California, USA, 2016–2018
- Macrolide-Resistant Mycoplasma pneumoniae Infections in Pediatric Community-Acquired Pneumonia
- Efficient Surveillance of Plasmodium knowlesi Genetic Subpopulations, Malaysian Borneo, 2000–2018
- Bat and Lyssavirus Exposure among Humans in Area that Celebrates Bat Festival, Nigeria, 2010 and 2013
- Rickettsioses as Major Etiologies of Unrecognized Acute Febrile Illness, Sabah, East Malaysia
- Meningococcal W135 Disease Vaccination Intent, the Netherlands, 2018–2019
- Risk for Coccioidiomycosis among Hispanic Farm Workers, California, USA, 2018
- Atypical Manifestations of Cat-Scratch Disease, United States, 2005–2014
- Paradoxal Trends in Azole-Resistant Aspergillus fumigatus in a National Multicenter Surveillance Program, the Netherlands, 2013–2018
- Large Nationwide Outbreak of Invasive Listeriosis Associated with Blood Sausage, Germany, 2018–2019
- High Contagiousness and Rapid Spread of Severe Acute Respiratory Syndrome Coronavirus 2
- Identifying Locations with Possible Undetected Imported Severe Acute Respiratory Syndrome Coronavirus 2 Cases by Using Importation Predictions
- Severe Acute Respiratory Syndrome Coronavirus 2–Specific Antibody Responses in Coronavirus Disease Patients
- Linking Epidemiology and Whole-Genome Sequencing to Investigate Salmonella Outbreak, Massachusetts, USA, 2018
- Burden and Cost of Hospitalization for Respiratory Syncytial Virus in Young Children, Singapore

- Human Adenovirus Type 55 Distribution, Regional Persistence, and Genetic Variability
- Policy Decisions and Use of Information Technology to Fight COVID-19, Taiwan
- Sub-Saharan Africa and Eurasia Ancestry of Reassortant Highly Pathogenic Avian Influenza A(H5N8) Virus, Europe, December 2019
- Serologic Evidence of Severe Fever with Thrombocytopenia Syndrome Virus and Related Viruses in Pakistan
- Survey of Parental Use of Antimicrobial Drugs for Common Childhood Infections, China
- Shuni Virus in Wildlife and Nonequine Domestic Animals, South Africa
- Transmission of Legionnaires’ Disease through Toilet Flushing
- Carbapenem Resistance Conferred by OXA-48 in K2-ST86 Hypervirulent Klebsiella pneumoniae, France
- Laboratory-Acquired Dengue Virus Infection, United States, 2018
- Possible Bat Origin of Severe Acute Respiratory Syndrome Coronavirus 2
- Heartland Virus in Humans and Ticks, Illinois, USA, 2018–2019
- Approach to Cataract Surgery in an Ebola Virus Disease Survivor with Prior Ocular Viral Persistence
- Clinical Management of Argentine Hemorrhagic Fever using Ribavirin and Favipiravir, Belgium, 2020

To revisit the July 2020 issue, go to: https://wwwnc.cdc.gov/eid/articles/issue/26/7/table-of-contents
Rapid, Sensitive, Full-Genome Sequencing of Severe Acute Respiratory Syndrome Coronavirus 2

Appendix

The following pages describe 2 validated protocols for generating high-quality, full-length severe acute respiratory syndrome coronavirus 2 genomes from primary samples. One protocol uses multiplex reverse transcription PCR, followed by MinION or MiSeq sequencing; the other uses singleplex, nested reverse transcription PCR and Sanger sequencing.
Protocols for SARS-CoV-2 sequencing

Pathogen Discovery Team
NCIRD/DVD/RVB
Centers for Diseases Control and Prevention
Table of Contents
Disclaimers ...............................................................................................................................4
Application Notes ......................................................................................................................5
Singleplex nested RT-PCR ..........................................................................................................6
  Protocol Notes ...........................................................................................................................6
  Required Reagents ....................................................................................................................6
  Procedure ..................................................................................................................................6
    1. First round of RT-PCR .........................................................................................................6
    2. Second round of semi-nested or nested PCR .......................................................................7
Sanger sequencing .......................................................................................................................8
  Required Reagents ....................................................................................................................8
  Procedure ..................................................................................................................................8
Multiplex PCR ............................................................................................................................9
  Protocol Notes ...........................................................................................................................9
  Required reagents .....................................................................................................................9
  Procedure ..................................................................................................................................9
    1. Generate primer pools .........................................................................................................9
    2. First-strand synthesis ...........................................................................................................9
    3. Multiplex PCR ....................................................................................................................10
Nanopore Sequencing ...............................................................................................................11
  Protocol Notes ........................................................................................................................11
  Procedure ................................................................................................................................11
    1. Barcode amplicons ..............................................................................................................11
    2. Prepare Nanopore Ligation-based Library .........................................................................12
    3. Load MinION and sequence .............................................................................................13
    4. Generate consensus sequences from MinION data .........................................................14
    5. Quality control and analysis suggestions .........................................................................15
Illumina Library Preparation and Sequencing .........................................................................16
  Protocol Notes ........................................................................................................................16
  Required Reagents ................................................................................................................16
  Procedure for Library Preparation ........................................................................................16
    1. Fragmentation and End Repair ........................................................................................16
    2. Adapter Ligation ...............................................................................................................17
    3. PCR enrichment of Adapter-Ligated DNA .......................................................................17
4. Sizing and quantitation

MiSeq sequencing

Protocol Notes

Required Reagents

Procedure

1. Dilute and Pool Libraries

2. Denature Libraries

3. Load and Run MiSeq

4. Generation of consensus sequences from MiSeq data

Appendix A – Singleplex PCR Primers

Appendix B – Sequencing Primers

Appendix C – Plate Setup for Nested PCR and Sanger Sequencing

Appendix D – Multiplex PCR Primers

Appendix E – AMPure XP bead clean-up

Appendix F – Quantitation using Qubit

Required reagents

Procedure

Appendix G – CENTRI-SEP 96 Protocol
Disclaimers

The findings and conclusions in this report have not been formally disseminated by the Centers for Disease Control and Prevention and should not be construed to represent any agency determination or policy.

The protocols described here are for research purposes only and should not be used in place of approved diagnostic testing.
Application Notes

Validation specimen submission extraction, and quantitation

1. For clinical specimens, CDC requested that submitting labs submit upper respiratory swabs in 2-3 mL viral transport medium (VTM), according to the guidelines detailed at: https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html.

2. Extraction of respiratory specimens in was performed using the QIAamp Viral RNA Mini Kit (QIAGEN). 200 uL of specimen VTM was used for each extraction and eluted from the column in 100 uL RNase-free water.

3. Samples for validation were quantitated with CDC N2 qRT-PCR assay, detailed at https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html

General Guidelines

1. Multiplex PCR Protocol is effective for Ct < 33.
2. Singleplex PCR is effective for some samples up to Ct 35. These are also useful for fill in reactions.
3. For the full Singleplex/Sanger protocol, two nucleic acid extractions (400 uL raw sample) will be needed.
Singleplex nested RT-PCR

Protocol Notes
To complete this protocol, 190 uL of extracted template is needed. For samples between Ct 27 and 35, two rounds of nested RT-PCR are recommended; for samples up to Ct 27, one round of RT-PCR is recommended. The resulting PCR products can be individually proceeded with Sanger sequencing, or they can be pooled for Oxford Nanopore or Illumina sequencing, depending on the number of samples and availability of sequencing platforms.

See Appendix C for recommended plate setups.

Required Reagents

| Company            | Product                                              | Catalog number |
|--------------------|------------------------------------------------------|----------------|
| Thermo (Invitrogen)| Superscript III one-step RT-PCR with Platinum Taq High Fidelity DNA polymerase | 12574035       |
| Sigma Aldrich (Roche)| Protector RNase inhibitor                           | 3335402001     |
| Takara             | LA Taq DNA polymerase with GC buffer                 | RR02AG         |
|                    | Nuclease Free water                                  |                |
|                    | 50 uM Primers                                        |                |

Procedure

1. First round of RT-PCR
   1.1. Prepare the first-round master mix as below. Please note, the protocol is generic as all 38 primer pairs require the same master mix (see Appendix A). For each SARS-CoV-2 sample to be sequenced, 38 individual PCR reactions are required.

   Component                              | Volume (uL) |
   ----------------------------------------|-------------|
   Water                                   | 1.75        |
   2x Buffer (2.4mM MgSO₄)                  | 12.5        |
   5mM MgSO₄                               | 4.5         |
   50uM Primer For                         | 0.25        |
   50uM Primer Rev                         | 0.25        |
   RNase Inhib. 40U/uL                     | 0.25        |
   SSIII / Platinum Taq high fidelity      | 0.5         |
   Pre-mix                                 | 20          |
   Template (RNA)                          | 5           |
   **Total**                               | **25**      |

   1.2. Add 5uL of RNA template to each of the 38 PCR reactions. Spin tubes/plates down and proceed to PCR.

   1.3. Perform first round PCR with the cycling parameters as below.
2. Second round of semi-nested or nested PCR

2.1. After first round RT-PCR is complete, prepare the master mix for 2nd round of semi-nested or nested PCR as below. Please note, the protocol is generic as all 38 second round primer pairs require the same master mix. Primer information is located in Appendix A. For the 2nd round of semi-nested- or nested PCR, there are 38 individual PCR reactions for each sample to be sequenced.

| Component                  | Volume (µL) |
|----------------------------|-------------|
| Water                      | 5.75        |
| 2× GCBuffer I              | 12.5        |
| dNTP Mixture (2.5 mM each) | 4           |
| 50µM Primer For            | 0.25        |
| 50µM Primer Rev            | 0.25        |
| TaKaRa LA Taq™ (5 units/µl)| 0.25        |
| Pre-mix                    | 23          |
| Template (1R product)      | 2           |
| Total                      | 25          |

2.2. Add 2 µL of the corresponding first round PCR product to the second round PCR master mix. Spin tubes/plates down and proceed to PCR.

2.3. Perform second round PCR with the cycling parameters as below.

| 60°C     | 1 minutes          | Decrease 0.5 C°/sec |
|----------|--------------------|---------------------|
| 50°C     | 30 minutes         |                     |
| 94°C     | 15 seconds         |                     |
| 55°C     | 15 seconds         |                     |
| 72°C     | 1 minute           |                     |
| 72°C     | 7 minutes          |                     |
| 4°C      | Hold               |                     |

2.4. Following the completion of second round PCR, run 3 µL of all 38 PCR reactions on 1% agarose gels or fragment analyzer to check for amplification.
Sanger sequencing

Required Reagents

| Company                          | Product                                         | Catalog number |
|---------------------------------|-------------------------------------------------|----------------|
| Thermo (Applied Biosystems)     | ExoSap-It                                       | 78201.1.ML     |
| Thermo (Applied Biosystems)     | BigDye v3.1 cycle sequencing kit                | 4337455        |
| Princeton Separations           | Centri-sep 96 well plates                       | CS-963         |
|                                  | Nuclease Free water                             |                |
|                                  | 5 uM Primers                                    |                |

Procedure

1. Transfer 10 uL of each PCR reaction to new tubes/plate for ExoSap cleanup. Add 4 uL ExoSap-It to each PCR reaction (10 uL) and incubate at 37°C for 15 minutes, followed by 80°C for 15 minutes on a thermocycler.
2. Prepare sequencing master mix as below.
   Sequencing primers for each amplicon are listed in Appendix B

| Component                          | Volume (uL) |
|------------------------------------|-------------|
| Water                              | 5.5         |
| 2x Buffer                          | 2           |
| 5uM Primer                         | 1           |
| BigDye 3.1 enzyme                  | 1           |
| Pre-mix                            | 9.5         |
| Template (2R PCR product)          | 0.5         |
| Total                              | 10          |

3. Add 0.5 uL of corresponding ExoSap cleaned PCR product to each sequencing reaction mix. Spin tubes/plates down and proceed to sequencing PCR.
4. Perform sequencing PCR with the parameters listed below:

| Temperature | Time   |
|-------------|--------|
| 96°C        | 2 minutes |
| 96°C        | 30 seconds |
| 50°C        | 15 seconds |
| 60°C        | 3 minutes |
| 4°C         | Hold    |

5. Following sequencing PCR, clean-up of sequencing reactions is performed with Centri-Sep 96-well plates following the manufacturer’s instructions (Appendix G) with one addition. 20 uL nuclease free water is added to the 96-well collection plate prior to the final spin.
6. The 96-well collection plate with the cleaned sequencing sample plus water is loaded onto the ABI sequencer.
7. Sequencher 5.4 is used for data analysis of Sanger PCR data.
Multiplex PCR

Protocol Notes
This protocol uses 10 uL of template for each sample. The pooled, multiplexed PCR products can be followed with nanopore sequencing or Illumina MiSeq sequencing depending on the number of samples and available sequencing platforms. We have been able to sequence full genomes reliably under Ct 30, and depending on the sample, up to Ct 33.

This protocol was adapted from Quick J et al. *Nat Protoc*. 2017 Jun;12(6):1261-1276.

Required reagents

| Company                  | Product                               | Catalog number |
|--------------------------|---------------------------------------|----------------|
| Thermo Fisher (Invitrogen) | SuperScript IV 1\textsuperscript{st} strand synthesis system | 18091200       |
| NEB                      | NEBNext Q5 Hot Start HiFi PCR Master Mix | M0543L         |
|                          | Nuclease Free water                   |                |
|                          | Primers                               |                |

Procedure

1. Generate primer pools
   1.1. Prepare primers as 50 uM primer stocks.
   1.2. Add an equal volume of each 50 uM primer stock to six 1.5mL Eppendorf tubes labeled as pool 1, 2, 3, 4, 5, and 6. Primers for each pool are listed in Appendix D.
   1.3. Prepare 10 uM working concentration by diluting each pool 1:5 with nuclease free water.

2. First-strand synthesis
   2.1. Mix the following components.

   | Component         | Volume (uL) |
   |-------------------|-------------|
   | RNA (template)    | 10          |
   | Random primer 25uM| 2           |
   | dNTPs             | 1           |
   | Total             | 13          |

   2.2. Denature the template-primer-dNTP mix at 65°C for 5 minutes.
   2.3. Place on ice for 5 minutes.
   2.4. Add the following components to the template-primer-dNTP mix:
| Component                  | Volume (uL) |
|----------------------------|-------------|
| 5x SSIV buffer             | 4           |
| 0.1 M DTT                  | 1           |
| RNase inhibitor            | 1           |
| SSIV RT (200 units/uL)     | 1           |
| **Total**                  | **20**      |

2.5. Incubate in a thermal cycler at the following temperatures:
- 25°C for 10 minutes,
- 50°C for 10 minutes,
- 85°C for 10 minutes, hold at 4°C.

2.6. Spin down. Can be stored at -20°C.
2.7. Add 1 uL RNase H and incubate at 37°C for 20 minutes.

3. **Multiplex PCR**
3.1. Mix the following components in 6 wells of a PCR plate or strip tube.

| Component                          | Volume (uL) |
|------------------------------------|-------------|
| NEBNext Q5 Hot Start HiFi PCR Master Mix | 15           |
| PCR grade water                    | 10.2        |
| Primer pool 1, 2, 3, 4, 5, or 6 (10uM) | 1.8          |
| **Total**                           | **27**      |

3.2. Add 3 uL of cDNA from above to each tube.
3.3. Run the following PCR program:

- **98°C** 30 seconds
- **98°C** 15 seconds 40 cycles
- 65°C 5 minutes
- 4°C Hold

Note: fewer cycles may be used, but 40 cycles is used to maximize detection of lower-titer samples.
3.4. Optional: Run a 2% agarose gel for each multiplexed PCR reaction pool 1, 2, 3, 4, 5, and 6 to check for specific bands of the correct size (0.4-0.6 kb).
3.5. Pool 20 uL from each of 6 tubes of multiplexed PCR reactions in a 0.3 mL tube in a PCR strip or a well in PCR plate (the total volume is 120 uL).
3.6. Add 1X ratio (120 uL) of AMPure XP beads to the PCR product pools.
3.7. Purify according to standard AMPure protocol (see Appendix E).
3.8. Elute in 80 uL water.
3.9. Quantitate 1 uL of cleaned PCR products using Qubit dsDNA HS kit (Appendix F).
3.10. Optional: Run a 2% agarose gel and load 3 uL of cleaned PCR products to check for specific bands of the correct size (0.4-0.6 kb).
Nanopore Sequencing

Protocol Notes
This protocol takes advantage of the multiplexing density afforded by the “PCR Barcoding Expansion 1-96” kit.
This protocol is derived from Oxford Nanopore’s protocols available at http://community.nanoporetech.com.

Required reagents for Nanopore barcoding and sequencing:

| Company | Product | Catalog number |
|---------|---------|----------------|
| NEB     | NEBNext Ultra II End-repair/dA tailing module | E7546 |
| NEB     | Blunt/TA ligase master mix | M0367 |
| NEB     | NEBNext Quick Ligation Module | E6056 |
| TaKaRa  | TaKaRa LA Taq DNA Polymerase with GC Buffer | RR02AG |
| Beckman Coulter | Agencourt Ampure XP Beads | A63880/A63881 |
| Oxford Nanopore Technologies | Nanopore Ligation Sequencing Kit (1D) | SQK-LSK109 |
| Oxford Nanopore Technologies | PCR Barcoding Expansion 1-96 | EXP-PBC096 |
| Oxford Nanopore Technologies | SpotON Flow Cell (R9.4.1) | FLO-MIN106D |
| Oxford Nanopore Technologies | MinION | MinION Mk1B |

Procedure

1. Barcode amplicons
   1.1. Mix the following components:
       
       | Component                     | Volume (uL) |
       |-------------------------------|-------------|
       | 500 ng amplicon DNA           | 25          |
       | Ultra II end-prep reaction buffer | 3.5        |
       | Ultra II end-prep enzyme mix  | 1.5         |
       | Total                         | 30          |

       1.2. Incubate at 20°C for 10 minutes, 65°C for 5 minutes, hold at 4°C.
       1.3. Add 1X ratio (30 uL) AMPure XP beads.
       1.4. Purify according to standard AMPure protocol (Appendix E).
       1.5. Elute the DNA target from the beads with 17 uL water.
       1.6. Optional: quantitate 1 uL of cleaned end-prep DNA using Qubit dsDNA HS kit (Appendix F)
       1.7. Mix the following components:

       | Component          | Volume (uL) |
       |--------------------|-------------|
       | Cleaned end-prep DNA | 15          |
       | Barcode Adapter    | 10          |
       | Blunt/TA ligase master mix | 25         |
       | Total              | 50          |

       1.8. Incubate at 20°C for 10 minutes.
       1.9. Add 1X ratio (50 uL) AMPure XP beads.
       1.10. Purify according to standard AMPure protocol (Appendix E).
       1.11. Elute the DNA in 12 uL water.
1.12. Transfer eluate into new PCR plate or well
1.13. Quantitate 1 uL of ligated DNA according to the protocol (Appendix F).
1.14. Mix the following components:

| Component                        | Volume(uL) |
|----------------------------------|------------|
| 30ng adapter-ligated DNA         | x          |
| PCR Barcode primer (one of BC1-BC96) | 1         |
| 2x GC Buffer I                   | 25         |
| dNTP mix (10mM)                  | 8          |
| TaKaRa LA Taq (5U/uL)            | 0.5        |
| Water                            | 50 – x     |
| **Total**                        | **50**     |

1.15. Mix by pipetting and spin down
1.16. Run the following PCR program:

| Temperature | Duration | Cycle |
|-------------|----------|-------|
| 95°C        | 3 minutes|       |
| 95°C        | 15 seconds| 18 cycles |
| 62°C        | 15 seconds|       |
| 72°C        | 1 minute  |       |
| 72°C        | 7 minutes |       |
| 4°C         | Hold     |       |

1.17. Add 1X ratio (50 uL) of AMPure XP beads.
1.18. Purify according to standard AMPure protocol (Appendix E).
1.19. Elute the DNA target from the beads with 25 uL water.
1.20. Quantitate 1 uL cleaned, barcoded PCR products with Qubit dsDNA HS kit (Appendix F).

2. Prepare Nanopore Ligation-based Library
2.21. Pool the barcoded PCR products equally by mass.
2.22. Prepare LSK109 ligation-based libraries by mixing the following components:

| Component                        | Volume(uL) |
|----------------------------------|------------|
| 1 ug pooled barcoded sample      | x          |
| DNA CS                           | 1          |
| Ultra II End-prep reaction buffer| 7          |
| Ultra II End-prep enzyme mix     | 3          |
| Water                            | 49-x       |
| **Total**                        | **60**     |

2.23. Incubate at 20°C for 10 minutes, 65°C for 5 minutes, hold at 4°C.
2.24. Add 1X ratio (60 uL) of AMPure XP beads.
2.25. Purify according to standard AMPure protocol (Appendix E).
2.26. Elute the DNA target from the beads with 62 uL water.
2.27. To ligate sequencing adapters, mixing the following components:
## Load MinION and sequence

3.33. Set up the MinION flow cell and host computer, including MinKNOW software.
3.34. Open the MinKNOW GUI from the desktop icon and establish a local connection.
3.35. Insert flow cell into MinION.
3.36. Click “Check Flow Cells” at the bottom of the screen then click “Start test.” Check the number of active pores available. When the check is complete, it is reported in the Notification panel. Check to ensure it has enough pores for a good sequencing run (warranty for flow cells: 800 nanopores or above checked within 5 days of receipt).
3.37. Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FB) at room temperature before placing the tubes on ice.
3.38. Thoroughly mix the Sequencing Buffer (SQB) and Flush Buffer (FB) tubes by vortexing.
3.39. Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.
3.40. Open the lid of the nanopore sequencing device and slide the flow cell’s priming port cover clockwise 90 degrees. (The following steps are demonstrated at [https://youtu.be/CC11Jlydqrc](https://youtu.be/CC11Jlydqrc))
3.41. Set a P1000 pipette to 200 µL, insert the tip into the priming port, turn the wheel until the dial shows 220-230 µL, or until you can see a small volume of buffer entering the pipette tip. Do not remove more than this.
3.42. Visually check that there is continuous buffer from the priming port across the sensor array.
3.43. Prepare the flow cell priming mix: add 30 µL of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by pipetting up and down.
3.44. Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles.
3.45. Wait for 5 minutes.
3.46. Thoroughly mix the contents of the Loading Beads (LB) by pipetting.
3.47. Prepare library for loading my mixing:

| Component                              | Volume (µL) |
|----------------------------------------|-------------|
| Sequencing Buffer (SQB)                | 37.5        |
| Loading Beads (LB), mixed immediately before use | 25.5        |
| 150-200 ng DNA Library                 | 12          |
| Total                                  | 75          |
3.48. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
3.49. Load 200 µL of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
3.50. Mix the prepared library gently by pipetting up and down just prior to loading.
3.51. Add 75 μL of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.
3.52. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.
3.53. Start the sequencing run using the MinKNOW software.

4. Generate consensus sequences from MinION data

There are many considerations for generating high-quality consensus data from the MinION. Here are some suggestions for basecalling based on our experience.

Software:

| Software          | Source URL                                      |
|-------------------|-------------------------------------------------|
| Guppy 3.4.1+      | https://community.nanoporetech.com/downloads    |
| Medaka 0.11.5     | https://github.com/nanoporetech/medaka           |
| Minimap2 2.17 (r941) | https://github.com/lh3/minimap2              |
| SAMtools 1.9      | http://www.htslib.org/                         |
| BCFtools 1.9      | http://www.htslib.org/                         |
| BAMClipper        | https://github.com/tommyau/bamclipper           |
| cutadapt 2.3+     | https://github.com/marcelm/cutadapt             |
| vcf_mask_lowcoverage.pl | https://github.com/CDCgov/SARS-CoV-2_Sequencing |
| IGV               | http://software.broadinstitute.org/software/igv/|

Example commands below have user-supplied variable names bold. You will need to customize the details to your environment.

4.1. Basecalling

Basecalling may also be done using MinKNOW software. If so, you may skip the Guppy basecalling step.

```bash
# Run Guppy
guppy_basecaller --input_path $rundir --save_path $outputdir -r 
--config na_r9.4.1_450bps_hac.cfg --barcode_kits EXP-PBC096 
--trim_barcode --require_barcode_both_ends
# Combine all the output fastq files
mkdir $outputdir/fastq
find $outputdir -name "*.fastq" |while read infile; do
  if [[ $i =~ barcode|unclassified ]]; then
    outfile=$(grep -Eo "barcode..|unclassified" <<< $infile).fastq
    outfile="fastq/$outfile"
    cat $infile >> $outfile
  fi
done
```

4.2. Filter on quality and length

Filtering out low quality sequence, as well as unexpectedly long and short reads helps tremendously on off-target mapping affecting consensus quality.
cutadapt -j $threads -m 300 -M 1200 -q 15 -o $fastqfiltered $fastqfile

4.3. Mapping
Download reference sequence from GenBank: MN908947.3

```bash
minimap2 -L -a -x map-ont -t 12 MN908947.fasta $fastqfiltered > $samfile
samtools view -b $samfile | samtools sort - -o $bamfile
samtools index $bamfile
```

4.4. Clip primers
This step requires a BEDPE file describing the positions of the primers. It is available at
[https://github.com/CDCgov/SARS-CoV-2_Sequencing](https://github.com/CDCgov/SARS-CoV-2_Sequencing)

BAMClipper by default will output at file with the suffix “primerclipped.bam.”
Clipping by position allows only primers near the beginning of a read to be trimmed (rather than
genuine sequence in the middle of a read), and it is faster than sequence-based trimming (e.g.
Porechop).

```bash
cd $outputdir
bamclipper.sh -b $bamfile -p SC2_200324.bedpe -n 12 -u 80 -d 80
```

4.5. Generate VCF and consensus sequences
Medaka is very lenient with calling variants. We generally require a variant quality score of >= 30 and
depth of coverage >= 20 to call a variant. Below 20X coverage, we call an ‘N.’
The script to automate the filtering is available at [https://github.com/CDCgov/SARS-CoV-2_Sequencing](https://github.com/CDCgov/SARS-CoV-2_Sequencing)

```bash
# Generate Medaka VCF File
medaka consensus --model r941_min_high_g344 --threads 12 \ $primerclippedbamfile
$primerclippedbamfile.hdf
medaka variant MN908947.fasta $primerclippedbamfile.hdf $vcf
# Filter variants and generate consensus sequence
vcf_mask_lowcoverage.pl --bam $primerclippedbamfile \ 
--reference MN908947.fasta --vcf $vcf --consout $consensusfasta \ 
--depth 20 --qual 30
```

5. Quality control and analysis suggestions
5.6. Watch out for 1-base insertions/deletions. Though consensus calling has improved considerably,
there are residual errors. There are several stretches in SARS-CoV-2 that have homopolymers long
efficient enough to be problematic.

5.7. Do not ignore other deletions. There have been several deletions reported (3, 9, 15, 33bp, 384bp,
etc.), so keep in mind the difference between a potential real indel and missing amplicon or nanopore
error.

5.8. IGV can be useful for examining the “believability” of variants. However, some of these 1-2bp indels
appear in the reads, but they cannot be confirmed by Illumina or Sanger sequencing. These are either
unlucky PCR bias or systematic sequencing error.
Illumina Library Preparation and Sequencing

Protocol Notes
Starting Material: 100 pg–250 ng DNA. We recommend that the DNA be in 1X TE (10 mM Tris pH 8.0, 1 mM EDTA), however, 10 mM Tris pH 7.5–8, low EDTA TE or water are also acceptable. If the input DNA is less than 26 µl, add TE (provided) to a final volume of 26 µl. This protocol is adapted from the NEBNext Ultra II FS protocol, which can be found in its entirety at http://www.neb.com.

For sizing, other devices, such as the 2100 BioAnalyzer, 5200 FragmentAnalyzer, QIAxcel, or LabChipGX may also be used. These vary in quantitation accuracy, so fluorometric quantitation with Qubit (or similar instrument) or qPCR is recommended.

Required Reagents

| Company                  | Product                                                                 | Catalog number |
|--------------------------|------------------------------------------------------------------------|----------------|
| New England Biolabs     | NEBNext Ultra II FS DNA Library Prep Kit for Illumina                   | E7805S/E7805L  |
| New England Biolabs     | NEBNext® Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) | E6440S/E6440L  |
| Beckman Coulter          | Agencourt Ampure XP Beads                                              | A63880/A63881  |
| Beckman Coulter          | 10mM Tris-HCl, pH 8.0                                                  |                |
| Beckman Coulter          | Molecular biology grade ethanol                                        |                |
| Beckman Coulter          | Nuclease-free water                                                    |                |
| Agilent                  | High Sensitivity D1000 screen tape                                      | 5067-5584      |
| Agilent                  | High Sensitivity D1000 reagents                                        | 5067-5585      |

Procedure for Library Preparation

1. Fragmentation and End Repair
   1.1. Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.
   1.2. Vortex the Ultra II FS Enzyme Mix 5-8 seconds prior to use and place on ice.
   1.3. Add the following components to a 0.2 ml thin wall PCR tube on ice

| Component                               | Volume (µL) |
|-----------------------------------------|-------------|
| NEBNext Ultra II FS Enzyme Mix (yellow tube) | 2           |
| NEBNext Ultra II FS Reaction Buffer (yellow tube) | 7           |
| DNA (pooled PCR amplicons)              | 26          |
| Total                                   | 35          |

1.4. Vortex the reaction for 5 seconds and briefly spin down. Place in a thermocycler with the heated lid set to ≥75°C and run the following program:

37°C for 7 minutes, 65°C for 30 minutes, 4°C hold indefinitely
2. Adapter Ligation

2.1. Determine dilution for adapter if necessary, see table below. Dilute the NEBNext Adapter for Illumina (red tube) in 10 mM Tris-HCl, pH 8.0 with 10 mM NaCl as indicated below.

| Input DNA in the End Prep reaction | Adapter dilution (volume of adapter: total volume) | Working adapter concentration |
|-----------------------------------|-----------------------------------------------|-------------------------------|
| 250 ng - 101 ng                   | No dilution                                   | 15 uM                         |
| 100 ng – 5 ng                     | 10-fold (1:10)                                | 1.5 uM                        |
| Less than 5 ng                    | 25-fold (1:25)                                | 0.6 uM                        |

2.2. Add the following components directly to the FS reaction mixture from 1.1(35 uL):

| Component                                      | Volume (uL) |
|------------------------------------------------|-------------|
| NEBNext Ultra II Ligation Master Mix (red tube) | 30          |
| NEBNext Ultra II Ligation enhancer (green tube) | 1           |
| NEBNext adapter for Illumina                   | 2.5         |

Notes:
- Mix the Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction.
- The Ligation master mix and ligation enhancer can be mixed ahead of time and is stable for at least 8 hours at 4°C. Do not premix the adapter prior to use in the adapter ligation step.
- The NEBNext adapter is provided in NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs)

2.3. Set a pipette to 50 uL and pipette entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note: The NEBNext Ultra II Ligation master mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance.

2.4. Incubate at 20°C for 15 minutes in a thermocycler with the heated lid off.

2.5. Add 3 uL of USER enzyme (red tube) to the ligation mixture.

Note: This step is only required for use with NEBNext adapters. USER enzyme is provided in NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs)

2.6. Mix well and incubate at 37°C for 15 minutes in a thermocycler with the heated lid set to ≥47°C

2.7. Add 57 uL (0.8X) re-suspended AMPure XP beads to the ligation reaction (87 uL).

2.8. Follow steps in the AMPure XP bead clean-up section (Appendix E).

2.9. Elute the DNA target from the beads by adding 17 uL of 10 mM Tris-HCl or 0.1X TE.

2.10. Transfer 15 uL to a new PCR tube for amplification.

3. PCR enrichment of Adapter-Ligated DNA

3.1. Add the following components to a sterile strip tube:
| Component                                      | Volume (uL) |
|------------------------------------------------|-------------|
| Adapter ligated DNA fragments (from above)     | 15          |
| Unique dual index primer pair*                 | 10          |
| NEBNext Ultra II Q5 master mix (blue tube)     | 25          |
| Total volume                                   | 50          |

*The primers are provided in NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs). Please refer to the NEB #E6440 manual for valid barcode combination and tips for setting up PCR reactions.

3.2. Set a pipette to 40 uL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

3.3. Place tube on a thermocycler and perform PCR amplification using the following PCR cycling conditions:

| Cycle step                      | Temperature | Time    | # of cycles |
|---------------------------------|-------------|---------|-------------|
| Initial denaturation            | 98°C        | 30 seconds | 1           |
| Denaturation                    | 98°C        | 10 seconds | 3-15*       |
| Annealing/extension             | 65°C        | 75 seconds |             |
| Final extension                 | 65°C        | 5 minutes | 1           |
| Hold                            | 4°C         | ∞        |             |

*Follow the recommendations for cycle number listed in the table below.

Cycle recommendations

| Input DNA in the end prep reaction | # of cycles required to generate a library yield of: |
|------------------------------------|--------------------------------------------------|
|                                    | 100 ng                                           |
|                                    | 250 ng                                           |
|                                    | 100 ng                                           |
|                                    | 50 ng                                            |
|                                    | 10 ng                                            |
|                                    | 5 ng                                             |
|                                    | 1 ng                                             |
|                                    | 0.5 ng                                           |
|                                    | 0.1 ng                                           |
|                                    | 1 ug                                             |
|                                    | 3-4                                              |
|                                    | 4-5                                              |
|                                    | 5-6                                              |
|                                    | 8-9                                              |
|                                    | 9-10                                             |
|                                    | 11-12                                            |
|                                    | 12-13                                            |
|                                    | N/A                                              |

3.4. Add 0.9X AMPure XP beads to the PCR reactions (45uL).

3.5. Follow steps in the AMPure XP bead clean-up section (Appendix E).

3.6. Elute DNA target from beads into 33 uL 0.1X TE.

3.7. Transfer 30 uL supernatant to a new PCR tube. Libraries can be store at -20°C.

3.8. Check size distribution of libraries and quantitate library concentration.

4. Sizing and quantitation

4.1. Allow TapeStation reagents to equilibrate at room temperature for 30 minutes prior to use.

4.2. Vortex reagents well before use.

4.3. To prepare ladder, mix 2 uL high sensitivity D1000 sample buffer with 2 uL high sensitivity D1000 ladder.
4.4. To prepare sample, mix 2 uL high sensitivity D1000 sample buffer with 2 uL sample.
4.5. Spin down, then vortex using IKA vortexer and adapter at 2000 rpm for 1 minute.
4.6. Spin down to position the sample at the bottom of the tube.
4.7. Load samples into the 2200 TapeStation instrument and follow the software procedure for analysis.
4.8. Quantitate 1 uL library sample with Qubit dsDNA HS kit (Appendix F).
MiSeq sequencing

Protocol Notes
This procedure requires Illumina-style libraries that have been quality-controlled and quantitated using the recommended procedures (i.e. TapeStation and Qubit or qPCR). Exact loading concentrations may vary by machine or lab-dependent factors. For more details on loading and running the MiSeq, consult the more detailed manuals at http://www.illumina.com.

Required Reagents

| Company   | Product                | Catalog number |
|-----------|------------------------|----------------|
| Illumina  | MiSeq reagent kit v3   | MS-102-3003    |
| Illumina  | PhiX control kit v3    | FC-110-3001    |
|           | NaOH                   |                |
|           | Nuclease-free water    |                |

Procedure

1. Dilute and Pool Libraries
   1.1. Calculate the molar concentration of each library to be diluted using average size from the TapeStation and mass from Qubit, using the following equation:

\[
\text{concentration (ng/µL)} = \frac{\text{mass (ng)} \times \text{library fragment size}}{660 \text{ g/mol}} \times 10^6 \text{ µL/L} = \text{concentration (nM)}
\]

   1.2.
   1.3. Make a 4nM dilution of each library.
   1.4. Combine equal volumes of each diluted library into a new tube. This is the 4nM library pool.

2. Denature Libraries
   2.1. Make a fresh dilution of 0.2N NaOH by combining the following volumes in a microcentrifuge tube:
   800 µL laboratory-grade water
   200 stock 1.0N NaOH
   2.2. Remove HT1 from freezer and thaw at room temperature. Store at 2°C to 8°C until you are ready to dilute denatured libraries.
   2.3. Combine the following volumes in a microcentrifuge tube:
   5 µL 4nM library
   5 µL 0.2N NaOH
   2.4. Vortex briefly and then centrifuge at 280 x g for 1 minute.
   2.5. Incubate at room temperature for 5 minutes.
   2.6. Add 990 µL pre-chilled HT1 to the tube containing denatured library. The result is 1 mL of a 20pM denatured library.
   2.7. Dilute the 20pM library to the desired concentration, see table below:

   | Concentration | 6 pM | 8 pM | 10 pM | 12 pM | 15 pM | 20 pM |
   |--------------|------|------|-------|-------|-------|-------|
   | 20 pM library | 180 µL | 240 µL | 300 µL | 360 µL | 450 µL | 600 µL |
   | Pre-chilled HT1 | 420 µL | 360 µL | 300 µL | 240 µL | 150 µL | 0 µL |

   2.8. Invert to mix and then pulse centrifuge.
2.9. Dilute stock PhiX to 4nM by combining:
   2 uL 10 nM PhiX library
   3 uL 10 mM Tris-Cl, pH 8.5 with 0.1% Tween 20

2.10. Denature the PhiX control by adding the following volumes in a microcentrifuge tube:
   5 uL 4nM PhiX library
   5 uL 0.2N NaOH
   Remaining 4nM PhiX can be frozen and reused

2.11. Vortex briefly to mix and centrifuge at 280 x g for 1 minute.

2.12. Incubate at room temperature for 5 minutes.

2.13. Dilute denatured PhiX library to 20 pM by adding 990 uL pre-chilled HT1 to the PhiX tube. Invert to mix.

2.14. If using a MiSeq reagent kit v2, dilute 20 pM PhiX library to 12.5 pM by adding the following volumes in a microcentrifuge tube:
   375 uL 20pM denatured PhiX library
   225 uL pre-chilled HT1

2.15. Combine library and PhiX control according to the table below:

|                        |          |
|------------------------|----------|
| Denatured and diluted PhiX | 30 uL   |
| Denatured and diluted library | 570 uL |

2.16. Set aside on ice until you are ready to load it onto the reagent cartridge.

3. Load and Run MiSeq
   3.1. Thaw frozen reagents overnight at 4°C overnight or in a RT water bath.
   3.2. Mix reagents thoroughly by inverting several times. Inspect the bottom of reagent cartridge to ensure all liquids return to the bottom of each tube without any air bubbles.
   3.3. Using a 1000 uL pipette tip, piece the foil on position 17.
   3.4. Using a fresh 1000 uL pipette tip, transfer the denatured and library (with PhiX spiked) into position 17.
   3.5. Generate Sample Sheet using MiSeq Experiment Manager.
   3.6. Load MiSeq according to onscreen instructions in the MiSeq Control software.
4. Generation of consensus sequences from MiSeq data

The Illumina MiSeq provides very high-quality data, and consensus sequences may be generated by a variety of methods, including commercial tools such as Geneious and CLC Genomics Workbench. The procedure outlined here is a suggestion using free, open source tools.

| Software   | Source URL                                      |
|------------|------------------------------------------------|
| cutadapt 2.3+ | https://github.com/marcelm/cutadapt           |
| bowtie2    | https://github.com/BenLangmead/bowtie2        |
| seqtk      | https://github.com/lh3/seqtk                  |
| SAMtools 1.9 | http://www.htslib.org/                       |
| BCFtools 1.9 | http://www.htslib.org/                       |
| IGV        | http://software.broadinstitute.org/software/igv/|

4.1. Trim reads for quality (Q25+) and for adapters on both ends. Then trim primer sequences (a hard 30 bases on each end), keeping only sequences that are at least 75 bases. For reads <150 bases, this will need to be modified.

```
cutadapt -j $threads -g GTTTCCCAGTCACGATA -G GTTTCCCAGTCACGATA 
    -a TATCCTGTAGACCTCAACCTACAGACTTAGCTTCCGATCT 
    -A AGACTGTAGACCTCAACCTACAGACTTAGCTTCCGATCT

--interleaved $read1 $read2 | cutadapt -j $threads --interleaved -m 75 -u 30 
    -u -30 -U 30 -U -30 -o $read1.trim.fastq -p $read2.trim.fastq -
```

4.2. Map reads to reference sequence

```
bowtie2 --sensitive-local -p $threads -x MN908947
    -1 $read1.trim.fastq -2 $read2.trim.fastq -S $samfile
samtools view -b $samfile | samtools sort -o $bamfile
samtools index $bamfile
```

4.3. Call variants, generate consensus sequence. This will call positions covered by at least 100 reads.

```
samtools mpileup -aa -d 8000 -uf MN908947.fasta $bamfile | 
    bcftools call -Mc | tee -a $vcf | 
    vcfutils.pl vcf2fq -d 100 -D 100000000 | 
    seqtk seq -A - | sed '2~2s/[actg]/N/g' > $consensusfasta
```

## Appendix A – Singleplex PCR Primers

| Amplicon | 1st round | Sequence | Size | 2nd round | Sequence | Size |
|----------|-----------|----------|------|-----------|----------|------|
| PCR1 1F_209_1 | SC2M1-2_RIGHT_95 | GCTGACGGCGATCATCAGCAC | 756 | W1_2L_368 | TGGAGGAGGCTTTACTCAAGGC | 597 |
| PCR2 W1_2F_00826_1 | SC2M1-2_RIGHT_95 | AACACTTCTGTCGCTGCTTGTG | 904 | W1_2F_00850_2 | TACCTTCCTGATGGTAAAGAG | 853 |
| PCR3 W1_3F_01573_1 | SC2M1-2_RIGHT_95 | GGTGTGTTGGGTGTTCGTCG | 938 | W1_3F_01596_2 | AGGTCTTTAGAAGACCTTGT | 894 |
| PCR4 W1_4F_02387_1 | SC2M1-2_RIGHT_95 | CATTCTGCTGCAATGGGACCA | 942 | W1_4F_02402_1 | AAGGAAGTCTACCAAGAGTG | 903 |
| PCR5 W1_5F_03185_1 | SC2M1-2_RIGHT_95 | ATTCCTAGTGTCACCATGAC | 1014 | W1_5F_03208_2 | GATGTAGTACAAACACTTGG | 868 |
| PCR6 W1_6F_05045_1 | SC2M1-2_RIGHT_95 | CAGCACCCGACCTTATGATG | 968 | W1_6F_05073_2 | TTGGTATGCACATGATCAC | 932 |
| PCR7 W1_7F_04884_1 | SC2M1-2_RIGHT_95 | TTCTCACATTCACTAGATGTTG | 933 | W1_7F_04904_2 | ATGGTTGAGTTCATCCCTTTC | 893 |
| PCR8 W1_8F_05676_1 | SC2M1-2_RIGHT_95 | ATGACCTGCTTGCAAAATGTAAG | 977 | W1_8F_05699_2 | CGACTGATGACAAAGACGG | 933 |
| PCR9 W1_9F_06522_1 | SC2M1-2_RIGHT_95 | TACAGAGGGATGTCGACAC | 954 | W1_9F_06543_2 | AGACTCTAAGCTGCTTTAGTG | 919 |
| PCR10 W1_10F_07326_1 | SC2M1-2_RIGHT_95 | TGAGTACATTTCATT AAATTTTTGAG | 999 | W1_10F_07356_2 | TATGTGTTAAATTAATCTTG | 952 |
| PCR11 W1_11F_08120_1 | SC2M1-2_RIGHT_95 | GCCACGGCTGCAAAGGGGTTTGGT | 952 | W1_11F_08184_2 | GCTCTTTCTGACATTGGTAAC | 922 |
| PCR12 W1_12F_08996_1 | SC2M1-2_RIGHT_95 | CAGGACGCTTAAACACTTAGTAC | 980 | W1_12F_09017_2 | AAGTGCTAATTTTTAAAGTCG | 949 |
| PCR13 W1_13F_09831_1 | SC2M1-2_RIGHT_95 | GTCTCAAAATTCTTACACTTGG | 1005 | W1_13F_09850_2 | GATGTGTTAATCTTAACTTAC | 966 |
| PCR14 W1_14F_10686_1 | SC2M1-2_RIGHT_95 | GTTTATAAAATGGGAAACGTG | 984 | W1_14F_10708_2 | TCTTCAATTATGACCATCAG | 949 |
| PCR15 W1_15F_11527_1 | SC2M1-2_RIGHT_95 | GCCAGAGATTGTTTATTTATGTG | 911 | W1_15F_11527_2 | GCCAGAGATTGTTTATTTATGTG | 889 |
| PCR16 W1_16F_12311_1 | SC2M1-2_RIGHT_95 | CTAGATGCGAGAACAGCCGG | 929 | W1_16F_12327_2 | GGCGAGAACAGCTACTTACG | 892 |
| PCR17 W1_17F_13112_1 | SC2M1-2_RIGHT_95 | AATCTCTGCTGCGGAGACAC | 930 | W1_17F_13126_2 | GCAAACACATCAAATTTGGT | 902 |
| PCR18 W1_18F_13873_1 | SC2M1-2_RIGHT_95 | TACTTGTCATGTCGCTGACAC | 914 | W1_18F_13873_1 | TACTTGTCATGTCGCTGACAC | 914 |
| PCR19 W1_19F_14655_1 | SC2M1-2_RIGHT_95 | GCTTTCAACATCACTACGAGAGT | 902 | W1_19F_14670_2 | TTCTTCAACTACATCTTAGT | 879 |
| PCR20 W1_20F_15429_1 | SC2M1-2_RIGHT_95 | GTGGAAATGTCTGTTGTTCG | 971 | W1_20F_15441_2 | ATGTTGCGGGTTCACTATATG | 939 |
| PCR21 W1_21F_16221_1 | SC2M1-2_RIGHT_95 | GCATACTGTCATGTCGCTGAC | 919 | W1_21F_16291_2 | GCATCGATGACTATCCTTATG | 849 |
| PCR22 W1_22F_17065_1 | SC2M1-2_RIGHT_95 | ATTCCTACTCCGCGACCCAC | 970 | W1_22F_17082_2 | CCACCTGTTGCTGTTAAGAGT | 930 |
| PCR23 W1_23F_17881_1 | SC2M1-2_RIGHT_95 | CAATGCCAGCGCTCTCTTGG | 1019 | W1_23F_17901_2 | TGTAGTTAAAGAGATTTTAA | 978 |
| PCR24 W1_24F_18767_1 | SC2M1-2_RIGHT_95 | GCGTTGACACTCAGGACAC | 910 | W1_24F_18786_2 | AAGCGATACCTACAAACCAC | 879 |

23 v200603.1
| PCR Stage | Primer Name | Forward Primer | PCR Product Size | Reverse Primer | PCR Product Size |
|-----------|-------------|----------------|------------------|----------------|-----------------|
| PCR25     | W1_25F_19546_1 | CAGCTGGCTTTAGCTTGTGGG | 936 | W1_25R_20572_1 | CAGCTGGCTTTAGCTTGTGGG |
| PCR26     | W1_26F_20343_1 | CATACTACATTTGGTTTATACG | 965 | W1_26R_20313_1 | GATGATTTGCTTCTATCTTTGG |
| PCR27     | W1_27F_21204_1 | CAGCTGGCTTTAGCTTGTGGG | 895 | W1_27R_22099_2 | CAAGGTCAAGAAAAAGC |
| PCR28     | W1_28F_21976_1 | CAGCTGGCTTTAGCTTGTGGG | 979 | W1_28R_22993_1 | TTTAGTTAAAAATCTCTTC |
| PCR29     | W1_29F_22847_1 | CATACTACATTTGGTTTATACG | 931 | W1_29R_22886_1 | GATGATTTGCTTCTATCTTTGG |
| PCR30     | W1_30F_23704_1 | CAGCTGGCTTTAGCTTGTGGG | 906 | W1_30R_24610_2 | CTGAGTCTAATTTATGGT |
| PCR31     | W1_31F_24514_1 | CAGCTGGCTTTAGCTTGTGGG | 962 | W1_31R_25476_1 | CAAAATCTGAGGAGGATC |
| PCR32     | W1_32F_25357_2 | CAGCTGGCTTTAGCTTGTGGG | 1001 | W1_32R_25411_1 | CAAAATCTGAGGAGGATC |
| PCR33     | W1_33F_26241_2 | CAGCTGGCTTTAGCTTGTGGG | 874 | W1_33R_26335_2 | CAAAATCTGAGGAGGATC |
| PCR34     | W1_34F_26999_2 | CAGCTGGCTTTAGCTTGTGGG | 993 | W1_34R_27992_2 | CAAAATCTGAGGAGGATC |
| PCR35     | W1_35F_27875_1 | CAGCTGGCTTTAGCTTGTGGG | 980 | W1_35R_28885_2 | CAAAATCTGAGGAGGATC |
| PCR36     | W1_36F_28716_1 | CAGCTGGCTTTAGCTTGTGGG | 1008 | W1_36R_29724_1 | CAAAATCTGAGGAGGATC |
| PCR37     | W1_37F_29596_2 | CAGCTGGCTTTAGCTTGTGGG | 280 | W1_37R_29873_2 | CAAAATCTGAGGAGGATC |
| PCR38     | W1_38F_30436_1 | CAGCTGGCTTTAGCTTGTGGG | 490 | W1_38R_490_1 | CAAAATCTGAGGAGGATC |
Appendix B – Sequencing Primers

Sequencing primer to amplicon matrix

| PCR Product | Sequencing primers |
|-------------|---------------------|
| PCR1        | W1_2L_368 SC2M1-2_RIGHT_965 SC2M1-2_LEFT_445 SC2M1-1_RIGHT_574 |
| PCR2        | W1_2F_00850_2 W1_2R_01703_2 W1_4F_1067* W1_3R_1206* |
| PCR3        | W1_3F_01596_2 W1_3R_02490_2 W1_6L_1819 W1_5R_1969 |
| PCR4        | W1_4F_02404_2 W1_4R_03037_2 W1_9L_2948* W1_8R_3094* |
| PCR5        | W1_5F_03208_2 W1_5R_04175_2 W1_11L_3638* W1_10R_3792* |
| PCR6        | W1_6F_04073_2 W1_6R_05005_2 W1_13L_4307* W1_12R_4522* |
| PCR7        | W1_7F_04904_2 W1_7R_05797_2 W1_15L_5159* W1_14R_5299* |
| PCR8        | W1_8F_05699_2 W1_8R_06632_2 SC2M1-16_LEFT_6030 SC2M1-15_RIGHT_6172 |
| PCR9        | W1_9F_06543_2 W1_9R_07462_2 W1_20L_6877* W1_19R_7009* |
| PCR10       | W1_10F_07356_2 W1_10R_08308_2 W1_22L_7625* W1_21R_7771* |
| PCR11       | W1_11F_08164_2 W1_11R_09106_2 W1_25L_8669* W1_24R_8794* |
| PCR12       | W1_12F_09017_2 W1_12R_09966_2 W1_27L_9308* W1_26R_9459 |
| PCR13       | W1_13F_09850_2 W1_13R_10816_2 W1_29R_10593* W1_30L_10448* |
| PCR14       | W1_14F_10708_2 W1_14R_11657_2 W1_32L_11111* W1_31R_11251* |
| PCR15       | W1_15F_11527_2 W1_15R_12416_2 W1_34L_11808* W1_33R_11948* |
| PCR16       | W1_16F_12327_2 W1_16R_13219_2 W1_37L_12878* W1_35R_12700* |
| PCR17       | W1_17F_13126_2 W1_17R_14028_2 W1_39L_13600* W1_38R_13741* |
| PCR18       | W1_18F_13873_1 W1_18R_14787_1 W1_41L_14342* W1_40R_14503 |
| PCR19       | W1_19F_14670_2 W1_19R_15549_2 W1_43L_14960* W1_42R_15108* |
| PCR20       | W1_20F_15441_2 W1_20R_16380_2 W1_46L_16004* W1_44R_15773* |
| PCR21       | W1_21F_16291_2 21R_17140_1 W1_48L_16735* W1_46R_16490* |
| PCR22       | W1_22F_17082_2 W1_22R_18012_2 W1_50L_17424* W1_49R_17553* |
| PCR23       | W1_23F_17901_2 W1_23R_18879_2 W1_53L_18503* W1_52R_18667* |
| PCR24       | W1_24F_18786_2 W1_24R_19665_2 W1_55L_19277* W1_54R_19405* |
| PCR25       | W1_25F_19546_1 W1_25R_20482_1 W1_57L_20013* W1_56R_20146* |
| PCR26       | W1_26F_20356_2 W1_26R_21300_2 W1_59L_20656* W1_58R_20796* |
| PCR27       | W1_27F_21204_2 W1_27R_22099_2 W1_61L_21411* W1_60R_21562 |
| PCR28       | W1_28F_21996_2 W1_28R_22975_2 W1_64L_22457 W1_63R_22612* |
| PCR29       | W1_29F_22664_2 W1_29R_23795_2 W1_66L_23182* W1_65R_23308* |
| PCR30       | W1_30F_23704_2 W1_30R_24610_2 W1_69L_24259* W1_67R_24002* |
| PCR31       | W1_31F_24514_2 W1_31R_25476_2 W1_71L_24935* W1_70R_25075* |
| PCR32       | W1_32F_25357_2 W1_32R_26358_2 SC2M1-66_LEFT_25665 SC2M1-65_RIGHT_25790 |
| PCR33       | W1_33F_26241_2 W1_33R_27115_2 SC2M1-68_LEFT_26454 SC2M1-67_RIGHT_26590 |
| PCR34       | W1_34F_26999_2 W1_34R_27999_2 SC2M1-71_LEFT_27650 SC2M1-69_RIGHT_27432 |
| PCR35       | W1_35F_27875_1 35hR R2_28855 W1_81L_28414* SC2M1-71_RIGHT_28203 |
| PCR36       | W1_36F_28716_2 W1_36R_29724_2 SC2M1-75_LEFT_29344 SC2M1-74_RIGHT_29469 |
| PCR37       | W1_37F_29596_2 W1_37R_29973_2 |
| PCR38       | 0_1b W1_1R_490 |

*Primer sequence in table below.

SC2M1 primers are sourced from the multiplex primer set (Appendix D). Others are primers from Appendix A.
| Name       | Sequence                             | Name       | Sequence                             |
|------------|--------------------------------------|------------|--------------------------------------|
| W1_4F_1067 | GGAATGTCCAATTTGATTTCC               | W1_41L_14342 | TTGGAATGACAGATGCTCTG                |
| W1_3R_1206 | TGGTGCGATCATTTGAGTGAC               | W1_43L_14960 | TAAATGGGTAAAGCTAGACTTTATAT         |
| W1_9L_2948 | GGTATTTTATGAATGAGTATGAC             | W1_42R_15108 | GGTGGCGAGCTACTTCTTTTC              |
| W1_8R_3094 | ATGGCGCAACTCCTTGTTCC                | W1_46L_16004 | GATGGACCGTGTCTTTACATG             |
| W1_11L_3638 | GCTAGCAACTCAACACACTTTC             | W1_44R_15773 | GCTAGCACTAAGCTTTGAGTG             |
| W1_1OR_3792 | AGCTAAGTAGATTCAGTGGGCAAC         | W1_48L_16735 | GGGAATGTTGAACCTAGACCAAC              |
| W1_13L_4307 | GAACGTCATTACTGTGATGAGCC          | W1_46R_16490 | AGCAACAAATGAACAAATGAAGGG           |
| W1_12R_4522 | CACCATATAACGACACCCCTC          | W1_50L_17424 | GTGCATAATGCTGACCCCTTC             |
| W1_15L_5159 | TTAGATCTACACACACACATGCTC         | W1_49R_17553 | TTCGAAGGAACATGCTGTTCC             |
| W1_14R_5299 | CAGTGGCAAGATACAGTGTGTTAC       | W1_53L_18503 | AGGAATGCTTTGGAAGATGAGGG            |
| W1_20L_6877 | AGGTTCGAGCTATTTGTTCTAGAGGC        | W1_52R_18667 | CATAGCACAACAGTGGCCTCAG             |
| W1_19R_7009 | CAGGGTTTATGAGATTAAGGAAC          | W1_55L_19277 | TTGATGGTGTCAGTTGATG               |
| W1_22L_7625 | ATTTTGATACATATGGCAGTGAGTAC       | W1_54R_19405 | CCATGAGACTCATAGTGAGCTG             |
| W1_23L_7771 | GATGGACCGGACTTCTCTGACTG          | W1_57L_20013 | GACTTATTTGAATAGGCGCTGTAATG         |
| W1_25L_8669 | TTTCAAAGTAGAATCAGATAGAATACAGG     | W1_56R_20146 | AACTGGTTTTTACGCTTCTC              |
| W1_24R_8794 | TACCAACAGCGCTGCTAACC             | W1_59L_20656 | AACTGATAGCAACCTCTGTTGGC            |
| W1_27L_9308 | CAGGATTTTTTCTGTTGTTAGTG          | W1_58R_20796 | ATTTGCGACATTACATATTAGG            |
| W1_29R_10593 | GTTACCTCTTGAAGCTGTGCAAC         | W1_61L_21411 | CTTAACATTAAGGATGCTCTGTTATG        |
| W1_30L_10448 | CCAATTTCACTATATGGGTCTACTCC       | W1_62R_22612 | AAAACATAGCAACCTCTGTTGAC           |
| W1_32L_11111 | TGGGTATTTTGTCTGATGTTGGT         | W1_66L_23182 | TCTACCCTCAATGGTTAAACAGGC           |
| W1_33R_11251 | TACGCACTCACCAGACATACAGG         | W1_63R_23308 | CAAGTGCTTGTGATACAGAC              |
| W1_34L_11808 | TGGCAACCTGTATGTACAGAAGTGC        | W1_69L_24259 | ATGCADATGTTGCTTATAGGTTAATG        |
| W1_35R_11948 | TGGTGCACTGAGCGATAGGCCC          | W1_67R_24002 | TTGCTTGGTTTATGAGTCTG              |
| W1_37L_12878 | TCTATACAAAGTGGCAACACCTTGT        | W1_71L_24935 | ACTGTGAGTGTTGTTAATAGGATTGCACG     |
| W1_35R_12700 | AGACATGTGTGTGACTAGCAGAAAGCAGAAGC | W1_70R_25075 | TGGCGAGGATGGTCACAAATCAC            |
| W1_39R_13600 | GTCGCTTCCAGGAAGGAGCG            | W1_81L_28414 | AATCTGGCTTGGTATCACC              |
Appendix C – Plate Setup for Nested PCR and Sanger Sequencing

Primers are added to each PCR reaction (PCR1-PCR38) prior to adding RNA. The layout stays the same until sequencing reactions are run.

We recommend making PCR primer plates (R1 and R2) in the same format so that primers may be added by multichannel pipetting.
We recommend making sequencing primer plates as shown below, so that primers may be rapidly added to the sequencing reactions. Primer sequences may be found in Appendices A and D.

| SC2MI-2_RIGHT_96S | SC2MI-2_LEFT_44S | SC2MI-1_RIGHT_574 | SC2MI-1_LEFT_644 | W1_5F_06543_2 | W1_9R_07467_2 | W1_20L_6877 | W1_19R_7009 | W1_17F_13126_2 | W1_17R_14028_2 | W1_39L_13600 | W1_38R_13741 |
|-------------------|-------------------|-------------------|-------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| W1_2F_00850_2    | W1_2R_01073_2    | W1_4F_1067       | W1_3R_1206       | W1_10F_0756_2  | W1_10R_08308_2 | W1_22L_7625   | W1_21R_7771   | W1_18F_13873_1 | W1_18R_14787_1 | W1_41L_14342  | W1_40R_14503  |
| W1_3F_01596_2    | W1_3R_02400_2    | W1_6L_1819       | W1_5R_1969       | W1_11F_08184_2 | W1_11R_09106_2 | W1_23L_8669   | W1_24R_8794   | W1_19F_14670_2 | W1_19R_15549_2 | W1_43L_14960  | W1_42R_15108  |
| W1_4F_02406_2    | W1_4R_03307_2    | W1_6R_1948       | W1_8R_3054       | W1_12F_09037_2 | W1_12R_09466_2 | W1_27L_4808   | W1_26R_5405   | W1_20F_15441_2 | W1_20R_16380_2 | W1_46L_16004  | W1_44R_15773  |
| W1_5F_03208_2    | W1_5R_04175_2    | W1_11L_3638      | W1_10R_2592      | W1_13F_05850_2 | W1_13R_10816_2 | W1_29R_10593  | W1_30L_10448  | W1_21F_10629_1 | W1_21R_11741_0 | W1_48L_16735  | W1_46R_16490  |
| W1_6F_04073_2    | W1_6R_05005_2    | W1_13L_4307      | W1_12L_4057      | W1_14F_10708_2 | W1_14R_11657_2 | W1_32L_11111  | W1_31R_11251  | W1_22F_11782_2 | W1_22R_18012_2 | W1_50L_17424  | W1_49R_17553  |
| W1_7F_04904_2    | W1_7R_05797_2    | W1_15L_5159      | W1_14L_5299      | W1_15F_11527_2 | W1_15R_12416_2 | W1_34L_11808  | W1_33R_11948  | W1_23R_17912_2 | W1_23R_18879_2 | W1_53L_18503  | W1_52R_18667  |
| W1_8F_05699_2    | W1_8R_06363_2    | SC2MI-16_LEFT_6030 | W1_16F_12327_2 | W1_16R_13219_2 | W1_37L_12878   | W1_35R_12700  | W1_24F_18786_2 | W1_24R_19665_2 | W1_55L_19277  | W1_54R_19405  |

Sequencing Reaction – Plate 1
Sequencing Primer Location

| SC2MI-16_LEFT_6030 | W1_2S_20154_1 | W1_25R_19384_2 | W1_25L_20013 | W1_56R_20146 | W1_53F_26241_2 | W1_33R_27115_2 | SC2MI-68_LEFT_26454 | SC2MI-68_RIGHT_26590 |
|-------------------|----------------|----------------|--------------|--------------|----------------|-------------------|------------------------|------------------------|
| W1_25F_15546_1    | W1_25R_20442_2 | W1_57L_20013   | W1_56R_20146 | W1_33F_26241_2 | W1_33R_27115_2 | SC2MI-68_LEFT_26454 | SC2MI-68_RIGHT_26590 |
| W1_26F_20356_2    | W1_26R_21300_2 | W1_59L_20656   | W1_58R_20796 | W1_34F_26999_2 | W1_34R_27992_2 | SC2MI-71_LEFT_27650 | SC2MI-71_RIGHT_27432 |
| W1_27F_21204_2    | W1_27R_22099_2 | W1_61L_21411   | W1_60R_21562 | W1_35F_27875_2 | W1_35R_28085   | W1_83L_28414   | SC2MI-71_LEFT_28203  |
| W1_28F_21996_2    | W1_28R_22975_2 | W1_64L_22457   | W1_63R_22612 | W1_36F_28716_2 | W1_36R_29724_2 | SC2MI-75_LEFT_29344 | SC2MI-75_RIGHT_29469 |
| W1_29F_22864_2    | W1_29R_23795_2 | W1_66L_23182   | W1_65R_23308 | W1_37F_29596_2 | W1_37R_29873_2 | SC2MI-75_RIGHT_29469 |
| W1_30F_23764_2    | W1_30R_24610_2 | W1_69L_24259   | W1_67R_24092 | 0_1b          | W1_18R_490     |
| W1_31F_24514_2    | W1_31R_25476_2 | W1_71L_24935   | W1_70R_25975 |
| W1_32F_25357_3    | W1_32R_26318_2 | SC2MI-68_LEFT_25665 | SC2MI-68_RIGHT_25790 |

Sequencing Reaction – Plate 2
Sequencing Primer Location
| Appendix D – Multiplex PCR Primers |
|-------------------------------------|
| **Pool 1**                           |
| **PCR** | **Name** | **Sequence** |
| 1 | SC2M1-1_LEFT_31 | ACCAACAACTTTCGATCTCTTGT |
|  | SC2M1-1_RIGHT_574 | GTGTCACCAACACGCTACC |
| 5 | SC2M1-5_LEFT_1706 | TCTGCTTCACAAAGTCTGTGTTG |
|  | SC2M1-5_RIGHT_2266 | ACAGGCGACATTGTTCCAG |
| 9 | SC2M1-9_LEFT_3306 | TGGAAGATCTGATGCGTGACAG |
|  | SC2M1-9_RIGHT_3878 | GCACGGATCAATCAGGTTT |
| 13 | SC2M1-13_LEFT_4885 | TCTGTCACACCTCAGTGTAGG |
|  | SC2M1-13_RIGHT_5400 | GCACAAAGTTCAGCTTCACC |
| 17 | SC2M1-17_LEFT_6408 | CTAAGGAATGAGTGGAAAATCTCAGCA |
|  | SC2M1-17_RIGHT_6903 | GCCTCTGACAAATATGCCGACACT |
| 21 | SC2M1-21_LEFT_8004 | TTGTGATAGTGCCGGAAGTTG |
|  | SC2M1-21_RIGHT_8553 | ACCACCTTGCAAAAAACGCTG |
| 25 | SC2M1-29_LEFT_9551 | AATGCGAGATCTCAATGCTGTGTTG |
|  | SC2M1-29_RIGHT_11047 | AGTCCGAGATCTCAATGCTGTGTTG |
| 33 | SC2M1-33_LEFT_12557 | ATCCAAGCGGTTGATAGCGAT |
|  | SC2M1-33_RIGHT_13136 | TTGTGTCGCCCACTAGCTAG |
| 37 | SC2M1-37_LEFT_14103 | TTTTCAACAAACAGGCCAGG |
|  | SC2M1-37_RIGHT_14641 | GTGACGCTACAGAAAAACGAG |
| 41 | SC2M1-41_LEFT_15637 | AGAATAATACAGTGTGACACAGACAG |
|  | SC2M1-41_RIGHT_16208 | GCCTCTGACAAATATGCCGACACT |
| 45 | SC2M1-45_LEFT_17317 | AATGCACTTGCAGGACAGACAG |
|  | SC2M1-45_RIGHT_19703 | CCAGAAGTGAAGCTGATGGT |
| 49 | SC2M1-49_LEFT_18897 | TTGAAAGGTGTGATAGCTGACT |
|  | SC2M1-49_RIGHT_19267 | GCACACCCAAAAGCTAGGCAAGCA |
| 53 | SC2M1-53_LEFT_20554 | TCTGAATTTCTAAGTGTTCAAGAGTAAG |
|  | SC2M1-53_RIGHT_21144 | AGATCGACCTTTGTGTTAATAACACCAAC |
| 57 | SC2M1-57_LEFT_22203 | GTGATCTCCCTCCAGGTTTTTCCG |
|  | SC2M1-57_RIGHT_22697 | ACCTAAAAGTGAAAAATGATGCGGAAG |
| 61 | SC2M1-61_LEFT_23737 | AATCTCAACCAGTCTCATGACAAGAC |
|  | SC2M1-61_RIGHT_24231 | GCACAAAGTCCCAAACCAAAAG |
| 65 | SC2M1-65_LEFT_25214 | CTAGGTTTTTATAGTGCTGGTTGGTC |
|  | SC2M1-65_RIGHT_25790 | CATTGCCCAAGCAAGAACGAGC |
| 69 | SC2M1-69_LEFT_26877 | CTTTCAACGTGCGCAACCTCAGT |
|  | SC2M1-69_RIGHT_27432 | AGCCAGGTGTTATACGTGGCAAG |
| 73 | SC2M1-73_LEFT_28525 | TGGTGCTACCCGAAGGCTACG |
|  | SC2M1-73_RIGHT_29045 | GCCTCTGAGAAGCCTCAGCAGC |
| **Pool 2**                           |
| **PCR** | **Name** | **Sequence** |
| 2 | SC2M1-2_LEFT_445 | TTTGCCCTCAACTTGGAACAGC |
|  | SC2M1-2_RIGHT_965 | GTCAGCGCCAGCAAGACATAC |
| 6 | SC2M1-6_LEFT_2138 | AAACCCCTCTTCAGTTGCGGTTG |
|  | SC2M1-6_RIGHT_2642 | TTTCGAGCAACATAGGCGCTT |
| 10 | SC2M1-10_LEFT_3715 | AGCTGTATTTTTTGGTCTGAC |
|  | SC2M1-10_RIGHT_4262 | CCTGACCGCTGTAGCTTATT |
| 14 | SC2M1-14_LEFT_5258 | ACTCTATTTAATAGGCGCTAACTACG |
|  | SC2M1-14_RIGHT_5818 | AGACCGCTCTGATACCATAAAAG |
| 18 | SC2M1-18_LEFT_6748 | AAACCGTGTGTTGACTATATATGGC |
|  | SC2M1-18_RIGHT_7255 | TGCCAAAACACCTTGCTAC |
| 22 | SC2M1-22_LEFT_8407 | CGTAAAGATTTCTATGCTTGGC |
|  | SC2M1-22_RIGHT_8913 | TGCAGAAGGTGGCGCTGAC |
| 26 | SC2M1-26_LEFT_9903 | AGTACAAAGTATTTAGGAGGACAGTG |
|  | SC2M1-26_RIGHT_10451 | TGGCCCTCATAGCAGTGGTA |
| 30 | SC2M1-30_LEFT_11400 | TGAATGTCATTGAACCTGTTATTAAGGTT |
|  | SC2M1-30_RIGHT_11944 | CTGAAACATGCTGCGCGCAAC |
| 34 | SC2M1-34_LEFT_13006 | TGCCACAGTCAGTCTCAGCTAG |
|  | SC2M1-34_RIGHT_13501 | GTGTAAGAGGCTGCGCTTAC |
| 38 | SC2M1-38_LEFT_14480 | ACTCAGAAGATGCTGTTGGA |
|  | SC2M1-38_RIGHT_15027 | TGCCAAAAGTTGCTTGTAC |
| 42 | SC2M1-42_LEFT_16065 | GGAATGAGTGGTGATGCTTTATG |
|  | SC2M1-42_RIGHT_16468 | GCCTCTGCTGCAGAAAAATTTG |
| 46 | SC2M1-46_LEFT_17752 | TTGGAAGAAAGTTGCTCTTTATCAC |
|  | SC2M1-46_RIGHT_18275 | GTCCTCTGCGGTTGAACAA |
| 50 | SC2M1-50_LEFT_19311 | TGTACCCACCAACACAGCTT |
|  | SC2M1-50_RIGHT_19866 | ATTACAGAACTACCCAC |
| 54 | SC2M1-54_LEFT_20990 | TGAATGTGTGGTGCACTAGTACA |
|  | SC2M1-54_RIGHT_21562 | TGTCTGTGATTTGCTAAGACAAACAT |
| 58 | SC2M1-58_LEFT_22563 | ACTGTGCTTCCCTGTTGGAG |
|  | SC2M1-58_RIGHT_23128 | TGTGCTGATGGTACAGTAC |
| 62 | SC2M1-62_LEFT_24095 | GCTGTGAGAACCTCACTTGTTG |
|  | SC2M1-62_RIGHT_24623 | AGCTGCTTATCTGGAGAGC |
| 66 | SC2M1-66_LEFT_25665 | CTCACACTTTTGGCTGTTGCT |
|  | SC2M1-66_RIGHT_26224 | GTTCGCAAGTCGAGCTTAC |
| 70 | SC2M1-70_LEFT_27254 | TTATGAGAGCTTATTTGGTCTAGG |
|  | SC2M1-70_RIGHT_27808 | AGCAGAAAAAGCTTAAAAGAC |
| 74 | SC2M1-74_LEFT_28918 | TGTACGTCTTCTGTTGTCTG |
|  | SC2M1-74_RIGHT_29469 | TGTGACGAGAAAGAAGAGCA |
| 78 | SC2M1-52_LEFT_20349 | AGCACGTAGTTGCTTGTACCTCAGAC |
|  | SC2M1-52_RIGHT_20798 | TTTGCGACATTCATCATATTAGCCT |
### Pool 3

| PCR Name       | Sequence                                      |
|----------------|-----------------------------------------------|
| SC2M1-3_LEFT_827 | AACACCTCTGTTGGCCCTGATG                      |
| SC2M1-3_RIGHT_1395 | TCTGATATGCTAGACTGTCAGACA                  |
| SC2M1-11_LEFT_4126 | GGGTGATGATTGTTGCAAGAGGT                    |
| SC2M1-11_RIGHT_4658 | ACCCGGGCGCTTACCCCTTCCGAGTGT                |
| SC2M1-15_LEFT_5677 | TGGTTGGAGATGATGAGCTGCTGATG                 |
| SC2M1-15_RIGHT_7964 | AGGCAACAGCTACAGCTTCAGATCATA               |
| SC2M1-19b_LEFT_7235 | TGGAGAAGGGTGATGAGCTGCTGAGT                 |
| SC2M1-19_RIGHT_7694 | AGCAGCGCTCTGTCAGTCTGCTGAC                 |
| SC2M1-23_LEFT_8778 | TATGGACGGCTGTTGATGAGTTA                    |
| SC2M1-23_RIGHT_9330 | TCTACACCACTACCCTTACATGCGA                 |
| SC2M1-27_LEFT_10318 | GCTTAAGGGTGATGAGCTGCTGAGT                 |
| SC2M1-27_RIGHT_10831 | AAGGGAAACTCCTTCTCAAGAGAAATGCC             |
| SC2M1-31_LEFT_11810 | GGGCAACCTTGTAGCAAGAGCTC                    |
| SC2M1-31_RIGHT_12335 | TGGCCCTCTGCTTCCAGTACGT                    |
| SC2M1-35_LEFT_13366 | AAACACACCTCTGAGCTGTCCGAC                   |
| SC2M1-35_RIGHT_13861 | TGTCAAAATCCTTCTACCAAAATGCC               |
| SC2M1-39_LEFT_14888 | ACAGTGTTGAGCTGTTAATAGCT                   |
| SC2M1-39_RIGHT_15391 | GGTGTGACAAGCTACAGCTC                     |
| SC2M1-43_LEFT_16518 | AAATACATGTTGTTGGTGGAGGGAGAATAGGT          |
| SC2M1-43_RIGHT_17087 | GGTGCTCTGCTGAGTAAGAT                    |
| SC2M1-47_LEFT_18148 | GGGTTATGCTGCTGACATCCTGAGCA                |
| SC2M1-47_RIGHT_18668 | CATAGACACAGGTGGCGCTCA                      |
| SC2M1-51_LEFT_19725 | TGAATGTTGCTATGAGTATGCTGTGTTAATAAA       |
| SC2M1-51_RIGHT_20162 | AGGGAACTCCTGCTTAAGAGGTAA                    |
| SC2M1-55_LEFT_21421 | CCGGAGGACGACCTTCTGATGG                     |
| SC2M1-55_RIGHT_21916 | CCCCTTACAAACGCTGCTGAC                     |
| SC2M1-59_LEFT_22986 | AAGGTAGTACCTCTTCTGCTGGTGGACAAA           |
| SC2M1-59_RIGHT_23519 | TGGATTATACCTAGTGTTGGACAGAA                 |
| SC2M1-63_LEFT_24493 | AAAATGGTGTGATGAGCTGAGAGAGAAGAC           |
| SC2M1-63_RIGHT_25003 | TGGATTAGTTTTGCTGAGAGAGAAGAC               |
| SC2M1-67_LEFT_26096 | AAATTGGTTGATGAGCTGAGAGAAGAC             |
| SC2M1-67_RIGHT_26590 | ACTAGGGTTTTGCTGAGAGAGAAGAC                |
| SC2M1-71_LEFT_27650 | TGGTTACAGACAGAACAGAGAGAAGAC             |
| SC2M1-71_RIGHT_28203 | AGGAAACGAGCCGACAGCTGAGAC                 |
| SC2M1-75_LEFT_29344 | TGGAGGATACACCAAAACAGCTCCAC               |
| SC2M1-75_RIGHT_29848 | AAAATACAGTGGGATAGACAGT                   |

### Pool 4

| PCR Name       | Sequence                                      |
|----------------|-----------------------------------------------|
| SC2M1-4_LEFT_1262 | ACCGGCAATTTTGGTAAAGCC                       |
| SC2M1-4_RIGHT_1840 | TCACAAATATCCGAGACACCTTT                    |
| SC2M1-8_LEFT_2932 | ACTTACAGACCTGGGAGCGATTT                     |
| SC2M1-8_RIGHT_3461 | CTGCAAACCTCTTCTCATGT                    |
| SC2M1-12_LEFT_4519 | TGTTGCTGATTTTGAATCTTACAGCTT              |
| SC2M1-12_RIGHT_5017 | CACAACTTGAGTGGAGAAGGT                     |
| SC2M1-16_LEFT_6030 | ACGGCAAGCTGAGTGAATTTAGTTG                 |
| SC2M1-16_RIGHT_6545 | TGATGCGCCACATCTCTTCTCTGT                  |
| SC2M1-20_LEFT_7560 | GGCTGTGTTTCTGACATGCTGCGGA                |
| SC2M1-20_RIGHT_8128 | TCGAGTTTGAGCTGCTGACA                      |
| SC2M1-24_LEFT_9203 | GATTCCTGAGTCTGAGGAGGGG                   |
| SC2M1-24_RIGHT_9734 | AGAACATGAAATGGGATGGAAAGAAGAGGGG        |
| SC2M1-28_LEFT_10697 | GGGAGACAGGTGTTTCTTCACTG                  |
| SC2M1-28_RIGHT_11209 | AGTCAAGTGGCGAGAGGAAGGT                    |
| SC2M1-32_LEFT_12201 | AGTTGAAAGTCTTCTGAGGAGGCT                 |
| SC2M1-32_RIGHT_12719 | CTGCAAGTCTGAGGAGGGAGGT                 |
| SC2M1-36_LEFT_13727 | GCTGTGCTGACTCGAGGAGG                     |
| SC2M1-36_RIGHT_14232 | AGGCTTTGTTACCTGAGGAGG                     |
| SC2M1-40_LEFT_15264 | TTAGAAGAAAACCATCCCTTTAATTTAGGT          |
| SC2M1-40_RIGHT_15771 | AGACATAGACCTCTGAGTAGA                     |
| SC2M1-44_LEFT_16948 | CTTACACTGAGCTGAGGAGG                     |
| SC2M1-44_RIGHT_17458 | GTGCAAGTATTGGGACGAGG                     |
| SC2M1-48_LEFT_18506 | GACCTTCTCGAGTATGAGGAGG                   |
| SC2M1-48_RIGHT_19038 | ACATACTGTTGAGGAGGAGG                     |
| SC2M1-52_LEFT_20124 | TGGAGAAGCTGAAGAAACAGCT                  |
| SC2M1-52_RIGHT_20698 | TGGAGAAGCTGAAGAAACAGCT                  |
| SC2M1-56_LEFT_21775 | TGGAGAAGCTGAAGAAACAGCT                  |
| SC2M1-56_RIGHT_22745 | ACCAGGCTGGCAAACCTGAGA                    |
| SC2M1-60_LEFT_23379 | ACCGGTTGCTGTTCTTTTACAGC                 |
| SC2M1-60_RIGHT_23976 | CAGTTAACTCACGCAAGACCCG                 |
| SC2M1-64_LEFT_24858 | GCAACACTGTTGAGGTAACAAA                 |
| SC2M1-64_RIGHT_25369 | TCGAAGAAGTTGCTGATGATGATGAGGT           |
| SC2M1-68_LEFT_26454 | TTTAAGACGCTTTGCTATTACTGTC              |
| SC2M1-68_RIGHT_27004 | AGTCAAGCTCCTCTTACAGT                  |
| SC2M1-72_LEFT_28066 | TTAATGTTGCTGAGAGAAGG                  |
| SC2M1-72_RIGHT_28649 | TAGACAACTAGGAGAGAAGG                |
| SC2M1-1_LEFT_2F2_1 | TTAAGGTTTATACCTCACCCAGG               |
| SC2M1-1_RIGHT_2G2_495 | CGAGCATCGAGAGAGAGAGTGA              |
This amplicon is intentionally doubled due to its reduced efficiency—add 2 parts of this primer set to the pool.

---

**Pool 5**

| PCR | Name | Sequence |
|-----|------|----------|
| 5W  | W1_S1_1457 | GTAGGGTGTGGCCTACATTAG |
| 5W  | W1_S5_1969 | GCTTAGGCCCTGCAGTCAAGAA |
| 81* | SC2M1_19a_LEFT_6057 | TGGTTTTTACATTAAAGTGTGCTAG |
| 81* | SC2M1_19a_RIGHT_7939 | TGGGGGACCATTGGCAAGATTT |
| 83  | SC2M1_21a_LEFT_7984 | AGGCTTATGCTGATGGTGGG |
| 28W | W1_28L_9659 | TGACACCTYTTAGTCTCTGAGTCAAC |
| 85  | SC2M1_34a_LEFT_12394 | CAGATGTAGTCTGCTAGTCAAG |
| 42  | SC2M1_42_LEFT_16065 | GAGTATGCTGTAGCTCTATTGTCAG |
| 89  | SC2M1_49a_LEFT_10112 | GGGTTTCTGCTGAAAGAGGAGAAAA |
| 91  | SC2M1_50a_LEFT_19181 | TGCTTTTGAAATCTTTATAGTTAG |
| 93  | SC2M1_51a_Left_19661 | TTTGATGAGAAGGATTGAGGAGAAG |
| 60W | W1_60L_21029 | GGATCCTATTAGTGGATCTAAGGAGG |
| 64W | W1_64L_22457 | CAGAACAGGTTACATCTTTTAGTTG |
| 98  | SC2M1_67a_LEFT_25910 | GGCACGACAGCTCTATTCTGCAAG |
| 100 | SC2M1_69a_LEFT_26846 | GCTAGCTCTTCTGCTGCAAGT |
| 102 | SC2M1_70a_LEFT_27262 | GAATGCTCTCTGCTGCAAGTTAG |
| 71  | SC2M1_71a_LEFT_27252 | TGGATGACGCAGGTTACATCTTT |
| 95  | 0_1b | TAAAGGGTATTACCTCTCTCGAGA |
| 36  | SC2M1_36_LEFT_13727 | GCTGTGCTAACAACTGACTTTCCTT |
| 36  | SC2M1_36_RIGHT_14232 | AGGCTTGTGTAAGCTGCTTGCAGAC |

---

**Pool 6**

| PCR | Name | Sequence |
|-----|------|----------|
| 6W  | W1_6L_1819 | AGGTGCCCTGGAATTGGTGCAAG |
| 6W  | W1_6R_2345 | AGTAGATGATGCAACCAAAAG |
| 7ab | SC2M1_7b_LEFT_2491 | AAGGAGAAGACCTCTCCCAAG |
| 84  | SC2M1_21b_LEFT_8240 | TCAAACTGACATAAAGGATCTGAG |
| 26W | W1_26L_8999 | TCTTGTTTTGCTGCTGAAAG |
| 40W | W1_40L_13986 | TGGCTCACCTGCTTGGCAG |
| 86  | SC2M1_34b_LEFT_13245 | CTGACTCCGTGTTGACCATAG |
| 90  | SC2M1_49b_LEFT_18955 | TGGCGCTGTAAGAGTTCAG |
| 92  | SC2M1_50b_LEFT_19395 | AGTCACATGAAACAAAGAGAGG |
| 94  | SC2M1_51b_LEFT_19957 | AAGCATTGTCGACCAAGCAG |
| 101 | SC2M1_69b_LEFT_26128 | TGCTGCTGCAAGTTGAGCATGAG |
| 101 | SC2M1_69b_LEFT_27443 | GAGTCACAAAGGAGGAGT |
| 9  | SC2M1_9_LEFT_3306 | TGGACACTACACACATGCTGAG |
| 75  | SC2M1_75_LEFT_29344 | TGGGTTGTGCAAAAGGAGG |
| 70b | SC2M1_70b_LEFT_27497 | TCTTCCTGAGGACAGAGAG |
| 95  | 0_1b | TTAAGGGTATTACCTCTCTCGAGA |
| 19-3| SC2M1_19a_LEFT_7235 | TCGACAGTCTGGTTCTGGCATATTCT |
| 62-2| SC2M1_62a_LEFT_23939 | ACCACAAAGAGGGTACCATATTG |

---

*This amplicon is intentionally doubled due to its reduced efficiency—add 2 parts of this primer set to the pool.*
Appendix E – AMPure XP bead clean-up

Bead-based clean-ups are done at several steps throughout the protocols presented. This covers the basic clean-up steps, make sure to check the specific protocol for the ratio of beads to use.

Depending on the number of samples, the AMPure XP bead clean-up takes about 30-40 minutes.

Required reagents for bead-based clean-up

| Company         | Product                  | Catalog number |
|-----------------|--------------------------|----------------|
| Beckman Coulter | Agencourt AMPure XP beads| A63882         |
|                 | 10mM Tris-HCl pH 8.0     |                |

1. Allow AMPure XP beads to warm to room temperature for at least 30 minutes before using.
2. Vortex AMPure XP beads to re-suspend.
3. Add appropriate ratio of re-suspended AMPure XP beads to the ligation reaction. Mix well by pipetting up and down at least 10 times.
4. Incubate for 5 minutes at room temperature.
5. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.
6. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (do not discard beads).
7. Add 200 uL of freshly prepared 80% Ethanol to the tube/plate while in the magnetic stand.
8. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant.
9. Add another 200 uL of freshly prepared 80% Ethanol to the tube/plate while in the magnetic stand.
10. Incubate at room temperature for 30 seconds, then carefully remove and discard the supernatant.
11. Air dry the beads for 2 minutes while the tube/plate is on the magnetic stand and with the lid(s) open.
   Caution: Do not over dry the beads. This may result in lower recovery of DNA target.
12. Remove the tube/plate from the magnet. Elute the DNA target from the beads by adding appropriate volume of 10mM Tris-HCl or water.
13. Mix well by pipetting up and down or on a vortex mixer. Incubate for 2 minutes at room temperature. If necessary, quickly spin the sample to collect liquid from the sides of the tube or plate wells before placing on the magnetic stand.
14. Place the tube/plate on the magnetic stand.
15. After the solution is clear (about 5 minutes), transfer to a new tube.
Appendix F – Quantitation using Qubit

Quantitation is done at several various steps throughout the protocols included and this protocol can be used anytime quantitation is indicated.

Required reagents

| Company        | Product                        | Catalog number |
|----------------|--------------------------------|----------------|
| Thermo Fisher  | dsDNA HS assay kit             | Q32854         |
| Thermo Fisher  | dsDNA BR assay kit             | Q32850         |
| Thermo Fisher  | Qubit assay tubes              | Q32856         |

Note: depending on the sample, either the high sensitivity (HS) or broad range (BR) kit may be used, the protocols are the same the only difference is the reagents.

Quantitation takes about 10-20 minutes depending on the number of samples.

Procedure

1. Set up the required number 0.5 mL Qubit assay tubes for standards and samples. Note: the standards require two tubes.
2. Label tube lids. Do not label the side of the tube as this could interfere with the sample read.
3. Prepare the Qubit working solution by diluting the Qubit dsDNA HS reagent 1:200 in Qubit dsDNA HS buffer. Use a clean plastic tube each time you prepare Qubit working solution. Do not mix the working solution in a glass container.

   The final volume in each tube must be 200 μL. Each standard tube requires 190 μL of Qubit working solution and each sample tube requires anywhere from 180-199 μL. Prepare sufficient Qubit working solution to accommodate all standards and samples.

4. Add 190 μL of Qubit working solution to each of the tubes used for standards.
5. Add 10 μL of each qubit standard to the appropriate tube, mix by vertexing 2-3 seconds.
6. Add Qubit working solution to individual assay tube, mix by vertexing 2-3 seconds.

   Your sample can be anywhere from 1-20 μL. Add a corresponding volume of Qubit working solution to each assay tube: anywhere from 180-199 μL.

7. Add each sample to the assay tubes containing the correct volume of Qubit working solution, then mix by vertexing 2-3 seconds. The final volume in each tube should be 200 μL.
8. Allow all tubes to incubate at room temperature for 2 minutes.
9. Sample concentration can now be measured on the Qubit Fluorometer.
CENTRI-SEP 96 Protocol

CENTRI-SEP 96 plates must be allowed to equilibrate to room temperature before use. We recommend that the plates be removed from the refrigerator at the same time the sequencing reactions are initiated. This will allow sufficient time for the plates to warm.

1. Remove the adhesive foil from the bottom and then from the top of the CENTRI-SEP 96 plate.
2. Stack the CENTRI-SEP 96 plate on top of a 96-well wash plate and centrifuge at 1500 x g for 2 minutes. Use an external timer and start timing when the rotor has reached the set speed. Discard the liquid in the wash plate. The gel matrix in the wells should appear opaque at this point.
3. Transfer the samples (20 µL or less) to the individual wells in the CENTRI-SEP 96 plate, taking care to place the samples in the centers of the gel beds.
4. Stack the CENTRI-SEP 96 plate on top of a 96-well collection plate and centrifuge at 1500 x g for 2 minutes.
5. Remove the 96-well collection plate containing the cleaned samples and dry in a speed-vac equipped with the appropriate rotor. Alternatively the plate can be sealed for storage.