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RSV Illumina Whole Genome Sequencing

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We use this protocol and it's working

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Abstract

This is the Illumina sequencing protocol used with ARTIC RSV primers to sequence RSV samples in the Royal Infirmary of Edinburgh, which has been utilised in this virological post (https://virological.org/t/preliminary-results-from-two-novel-artic-style-amplicon-based-sequencing-approaches-for-rsv-a-and-rsv-b/918). Primer scheme details can be found on the ARTIC RSV github page (https://github.com/artic-network/artic-rsv).

This protocol is used for routine RSV sequencing here, but these RSV primers have been developed to slot in to any existing amplicon sequencing protocols used in your lab, and have been used successfully in a range of different labs (publication in preparation).

Guidelines

Here we outline the major sections of this protocol and some things to prepare before starting the section.

Reverse Transcription (RT) using LunaScript RT SuperMix

This step reverse transcribes the RNA fragments primed
with random hexamers into first strand cDNA using reverse transcriptase.
Add RT Supermix in the clean area then transfer to the pre-PCR area for addition of extracts.

Tiled Whole Genome PCR Amplification using ARTIC primers

This step uses two separate PCR reaction pools to amplify cDNA
Make PCR mastermix in the clean area and transfer to the pre-PCR area for addition of RT product.

Tagmentation of PCR amplicons

This step "tagments" PCR amplicons, a process that fragments and tags the PCR amplicons with adapter sequences.
Prepare the TAG plate in the post-PCR area.

Prepare the following consumables:

- EBLTS HT (fridge): Bring to room temperature. Vortex thoroughly before use.
- TB1 HT (freezer): Bring to room temperature. Vortex thoroughly before use.

Post Tagmentation Clean Up

This step washes the adapter-tagged amplicons before PCR amplification.
Perform the clean-up in the post-PCR area.

Prepare the following consumables:

- ST2 HT (room temperature): Dispense slowly to minimise foaming.
- TWB HT (fridge): Dispense slowly to minimise foaming.
- EPM HT (freezer): Place on ice to thaw for next step.

Amplify Tagmented Amplicons

This step amplifies the Tagmented amplicons adding a prepared 10 base pair Index1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation.
Prepare the TAG plate for PCR in the post-PCR area.

Prepare the following consumables:

- EPM HT (freezer): Invert to mix and keep on ice until use.
- Index adapter plate (freezer): Thaw at room temperature. Vortex to mix, then centrifuge at 1000 x g for 1 minute. To open each prepared index adapter plate seal, align a new 96-well PCR plate above the index adapter plate, then press down to puncture the foil seal. Discard the PCR plate and use a new PCR plate for each index adapter plate.

Pool and Clean Libraries

This step combines libraries from each 96-well sample plate into one tube. Libraries of optimal size are then bound to magnetic beads, and fragments that are too small or large are washed away.

Pool and clean up libraries in post-PCR area.

Prepare the following consumables:

- ITB (Illumina Tune Beads; room temperature): Vortex before each use and frequently to make sure beads are evenly distributed. Aspirate and dispense slowly due to viscosity of the solution.
- RSB HT (fridge): Let stand for 30 minutes at room temperature. Vortex and invert to mix.

Quantify and Normalize Libraries

Quantify the resulting library pool using the Qubit dsDNA HS Assay kit as detailed in the protocol.

Pool and dilute libraries

This step pools and dilutes libraries to the starting concentration for the NextSeq 2000.

Materials

Equipment

10 µl, 20 µl, 200 µl and 1000 µl pipettes (Starlab)
10 µl 8-channel pipette (Starlab)
100 µl 8-channel pipette (Starlab)
Mini plate spinner centrifuge (Fisher Scientific)
C1000 Touch™ Thermal Cycler (Bio-Rad)
Vortex mixer (Fisherbrand)
Centrifuge 5340 (Eppendorf)
Multi Plate Shaker (Grant-bio)
DynaMag™-96 Side Skirted magnet (Invitrogen)
Microcentrifuge (VWR)
Qubit 3.0 Fluorometer (Thermo Fisher)
NextSeq 2000 (Illumina)

Consumables

Hard-Shell 96-well
PCR plates (Bio-Rad)
10 µl, 20 µl, 200 µl and 1000 µl filtered pipette tips
1.5 ml
microcentrifuge tubes (alpha laboratories)
Microseal® 'B'
seals (Bio-Rad)
25 ml universal tubes (Sarstedt)
LoBind 2 ml tubes (Eppendorf)

Reagents

NEB *LunaScript* RT *Supermix Kit* (E3010)
IPM HT (Illumina PCR Mix HT)
ARTIC Primer Mix
TB1 HT (Tagmentation Buffer 1 HT)
EBLTS HT (Enrichment BLT HT)
Nuclease free water

ST2 HT (Stop Tagment Buffer 2 HT)
TWB HT (Tagmentation Wash Buffer HT)
EPM HT (Enhanced PCR Mix HT)
Index adapters (IDT for Illumina - PCR index sets 1-4)
ITB (Illumina Tune Beads)
RSB HT (Resuspension Buffer HT)
Freshly prepared 80% Ethanol
NextSeq 2000 P2 reagents (200 cycles) (Illumina)

Before start

This protocol requires nucleic acid extracts from RSV positive specimens. Nucleic acid extracts tested have been extracted using either the Biomerieux easyMAG or eMAG automated extractors.

Thaw reagent cartridge 9-16 hours before loading.

Check the Guidelines tab for details on each section and what reagents are need to prepared beforehand.

Reverse Transcription (RT) using LunaScript RT SuperMix

1 Invert extract tubes and pulse spin to 3000 rpm to remove drops from lids.

2 In clean area, label a PCR plate with RSV, date, run number, and “RT”.

3 Dilute 4 µL RT mix with 6 µL nuclease-free water per sample (for 96-well plate: 400 µL of RT mix and 600 µL water).

4 Add 10 µL of diluted RT mix into each well of the RT Bio-Rad plate.

5 Add 10 µL of extract to each well of the RT plate. Based on the plate layout in the run worksheet, identify the location of the negative and positive controls and add NTC (nuclease-free water) or RSV Positive Control, respectively.

6 Seal the plate, invert to mix, and pulse centrifuge to remove droplets from lids.

7 Load plate into thermal cycler and run as outlined in the table below.
Samples can be stored at 20 °C if not used immediately.

Step	Temp	Time	Cycles
Primer Annealing	25 oC	2 min	1
cDNA Synthesis	55 oC	10 min	
Heat Inactivation	95 oC	1 min	
Hold	4 oC	Hold	

Tiled Whole Genome PCR Amplification using ARTIC primers

8 In the clean area, thaw PCR reagents at room temperature.

9 Invert tubes and pulse spin to 3000 rpm to remove drops from lids.

10 Make 1:10 dilutions of ARTIC RSV Primer mix sets 1 and 2 in nuclease-free water.

11 Label two Bio-Rad PCR plates with RSV, date, run number, and “PCR”. Label one of the plates “P1” and the other “P2”.

12 Label 2 x 25 ml universal tubes; one ‘P1’ and one ‘P2’

13 Make two PCR mixes – one using primer mix 1 (P1) and one using primer mix 2 (P2).

	No. of samples	1	96
	Compnent	x1 (ul)	x100 (ul)
	IPM HT	15	1500
	ARTIC RSV Primer Mix set 1 or 2	4.3	430
	Nuclease free water	4.7	470

14 Add 20 µL of PCR mix P1 into PCR plate P1. Add 20 µL PCR mix P2 into PCR plate P2.

15 Seal plates and transfer to pre-PCR area.

16 Using a multichannel pipette, add 5 µL of RT product to the corresponding wells in PCR plates ‘P1’ and ‘P2’.

17 Seal the plates and centrifuge to remove droplets from lids.

18 Load the PCR plates into the thermal cyclers and run as outlined below.

	Step	Temp	Time	Cycles
	Heat Activation	98 oC	3 min	1

Step	Temp	Time	Cycles
Denaturation	98 oC	15 sec	35
Annealing	63 oC	5 min	
Hold	4 oC	Hold	

Samples can be stored at -20 °C if not used immediately.

Tagmentation of PCR amplicons

1m

19 Label a Bio-Rad PCR plate with RSV, date, run number, and 'TAG'.

20 Combine amplicons from Pool 1 and Pool 2 as follows:

20.1 Transfer 10 µL from each well of the P1 plate to the corresponding well of the TAG plate.

20.2 Transfer 10 µL from each well of the P2 plate to the corresponding well of the TAG plate.

21 In a 25 ml universal tube, combine the following volumes to prepare Tagmentation Master Mix:

No. of samples	1	96
Compnent	x1 (ul)	x100 (ul)
TB1 HT	12	1200
EBLTS HT	4	400
Nuclease free wa ter	20	2000

22 Add 30 µL master mix to each well in the TAG plate.

23 Seal and shake at 1600 rpm for 00:01:00 .

1m

24 Load the TAG plate into a thermal cycler and run as outlined below.

Step	Temp	Time	Cycles
Annealing	55 oC	5 mins	1
Hold	4 oC	Hold	

Post Tagmentation Clean Up

15m

25 Centrifuge the TAG plate at 500 x g for 00:01:00 1m

26 Add 10 µL ST2 HT to each well of the TAG plate.

27 Seal and shake at 1600 rpm for 00:01:00 . 1m

28 Incubate at room temperature for 00:05:00 . 5m

29 Centrifuge at 500 x g for 00:01:00 . 1m

30 Place on the magnetic stand and wait until the liquid is clear (00:03:00) 3m

31 Inspect for bubbles on the seal. If present, centrifuge at 500 x g for 00:01:00 , and then place on the magnetic stand (00:03:00). 4m

32 Remove and discard all supernatant.

33 Wash beads as follows:

33.1 Remove from the magnetic stand

33.2 Add 100 µL TWB HT to each well.

33.3 Seal and shake at 1600 rpm for 00:01:00 . 1m

33.4 Centrifuge 500 x g for 00:01:00 . 1m

33.5 Place on the magnetic stand and wait until the liquid is clear (00:03:00) 3m

34 Wash beads a second time. Leave supernatant in plate for second wash to prevent beads from over drying.

Amplify Tagmented Amplicons 2m

35 In a 15 ml tube, combine the following volumes to prepare the PCR Master Mix. Multiply each volume by the number of samples.

	No. of samples	1	96
	Compnent	x1 (ul)	x100 (ul)
	EPM HT	24	2400
	Nuclease free wa ter	24	2400

36 Vortex PCR Master Mix to mix.

37 Keep the TAG plate on magnetic stand and remove TWB HT.

38 Use a 10 µl multi-channel pipette to remove any remaining TWB HT.

39 Remove the TAG plate from the magnetic stand.

40 Add 40 µL PCR Master Mix to each well.

41 Using a 10 µl multi-channel pipette, transfer 10 µL index adapters from the index adapter plate to the corresponding wells of the TAG plate.

42 Seal and shake at 1600 rpm for 00:01:00 . 1m

43 If liquid is visible on seal, centrifuge at 500 x g for 00:01:00 . 1m

44 Inspect to make sure beads are resuspended. To resuspend, set your pipette to 35 µl with the plunger down, and then slowly pipette to mix.

45 Load the TAG plate on a thermal cycler and run as outlined below:

	Step	Temp	Time	Cycles
	Heat Activation	72 oC	3 min	1
	Initial Denaturation	98 oC	3 min	1
	Denaturation	98 oC	20 sec	7
	Annealing	60 oC	30 sec	
	Elongation	72 oC	1 min	
	Final Elongation	72 oC	3 min	1
	Hold	10 oC	hold	

Pool and Clean Librarie 4m

46 Centrifuge the TAG plate at 500 x g for 00:01:00 . 1m

47 Place on the magnetic stand and wait until the liquid is clear (00:03:00). 3m

48 Pool libraries as follows:

48.1 Use a 10 µl multi-channel pipette to transfer 5 µL library from each well of the TAG plate to one column of a new PCR plate. Change tips after each column. These volumes result in

60 µL pooled library per row.

48.2 Label a new LoBind Eppendorf tube ‘Pooled ITB’.

48.3 Transfer 55 µL pooled library from each well of the new PCR plate into the Pooled ITB tube. For one sample plate, these volumes result in 440 µL of pooled library.

49 Vortex the Pooled ITB tube to mix, and then centrifuge briefly.

50 Vortex ITB to resuspend.

51 Add 0.9 x pooled library volume of ITB to the Pooled ITB tube:

	No. of samples	96
	Library volume	440ul
	ITB	396ul

52 Vortex to mix.

53 Incubate at room temperature for 00:05:00 . 5m

54 Centrifuge briefly.

55 Place on the magnetic stand and wait until the liquid is clear (00:05:00) 5m

56 Remove and discard all supernatant.

57 Wash beads as follows:

57.1 Keep on the magnetic stand and add 1000 µL fresh 80% ethanol to each tube.

57.2 Wait 00:00:30 . 30s

57.3 Remove and discard all supernatant.

58 Wash beads a second time.

59 Use a 20 µl pipette to remove all residual ethanol.

60 Add 55 µL RSB HT.

61 Vortex to mix, and then centrifuge briefly.

62 Incubate at room temperature for 00:02:00 . 2m

63 Place on a magnetic stand and wait until the liquid is clear (00:02:00) 2m

64 Transfer 50 µL supernatant from the Pooled ITB tube to a new LoBind Eppendorf tube labelled with:
- Run number
- Index number
- ARTIC primer version
- Date

Safe stopping point – store at -20 °C

Quantify and Normalize Libraries 2m 6s

65 Make a 1:10 dilution of the library pool in RSB HT (5 µL library, 45 µL RSB HT).

66 Label the required number of 0.5 ml Qubit tubes for standards and samples. Qubit assay required 2 standards (S1 and S2).

67 Prepare Qubit working solution in a 5 ml Universal tube as follows:

	Component	x1 (ul)	x5 (ul)
	Qubit dsDNA HS Reagent	1	5
	Qubit dsDNA HS Buffer	199	995

68 Add 190 µL of Qubit working solution to tube S1 and S2.

69 Vortex each Qubit standard then add 10 µL to appropriate tube and mix by vortexing for 00:00:03 . 3s

70 Add 199 µL of Qubit working solution to sample tubes.

71 Add 1 µL of diluted sample to appropriate tube and mix by vortexing for 00:00:03 . 3s

72 Allow all tubes to incubate at room temperature for 00:02:00 . 2m

73 On the home screen of the Qubit 3.0 Fluorometer, press ‘DNA’ and select ‘dsDNA High Sensitivity’ assay type. Press ‘Read standards’.

74 Insert S1 tube into sample chamber, close lid and press ‘Read standard’.

75 Perform as above for S2.

76 Press ‘Run samples’, select sample volume as ‘1µl’ and units as ‘ng/µl’

77 Insert sample tube into sample chamber and read tube.

78 Calculate the molarity of the pooled libraries. Use 400bp as the average library size. The following formula can be used:

$$\frac{Library\ concentration\ ng/\mu l}{660\ \frac{g}{mol}\ x\ average\ library\ size\ (bp)} x10^6 = Molarity\ (nM)$$

Pool and Dilute Libraries

79 Dilute each library pool to a normalised concentration of [M] 4 nanomolar (nM) in 60 µL using RSB HT.

80 For each set of 384 samples, combine 15 µL of each normalised pool containing index adapter set 1, 2, 3 and 4 in a new microcentrifuge tube. This step produces a final pool of 384 samples diluted to a starting concentration of [M] 4 nanomolar (nM) .

81 Further dilute down the combined library to a loading concentration of [M] 1 nanomolar (nM) ([M] 1000 picomolar (pM)) in RSB HT (25 µL in 100 µL) including 1% PhiX control in a new microcentrifuge tube.

Prepare for Sequencing

82 Invert cassette 10 times. (This assay requires a 200 cycle reagent kit).

83 Insert flow cell.

84 Pierce foil on loading port circled in diagram below and load 20 µL library.

Set Up Sequencing Run (NextSeq2000)

85 Select menu bar for settings.

86 Select settings.

87 Ensure local run set up is selected and choose output folder.

88 Select Start and start with sample sheet.

89 Select sample sheet for Run and review.

90 Ensure correct settings are selected and then choose prep.

91 Load flow cell into reagent cartridge and then into NextSeq2000.