IDbyDNA

Explify®

Respiratory Pathogen ID/AMR Panel

User Guide

Sample Preparation and Sequencing Library Preparation

NOTICE: This document does not, and is not intended to, exhaustively set forth safety procedures or instructions that would be necessary and appropriate in order to safely perform the procedures set forth herein. It is advisable that the procedures and protocols in this document be followed only by qualified and properly trained personnel in observance of all appropriate safety procedures, including but not limited to the use of appropriate personal protective equipment.

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1. Overview

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Overview

1.1 Introduction

Next-generation sequencing (NGS) provides an effective way to analyze samples and detect known and emerging respiratory pathogens from various sample types, including those with multiple infectious agents, in a single assay. The >2,000 total targets of the Illumina/IDbyDNA Respiratory Pathogen ID/AMR Panel (RPIP) encompass viruses, bacteria, and fungi, and associated antimicrobial resistance (AMR) markers. Combining RPIP with Illumina RNA Prep with Enrichment and sequencing on any Illumina sequencer, with subsequent data analyses using the IDbyDNA Explify platform offers several advantages, including:

- Sensitive detection of both DNA- and RNA-based respiratory pathogens in a single assay
- Comprehensive genome coverage of SARS-CoV-2 and Influenza A/B viruses
- Concurrent profiling of AMR marker expression for pathogen characterization in the same assay

This user guide outlines a streamlined laboratory workflow, from processing clinical samples for nucleic acid extractions, to preparing indexed libraries, followed by enrichment of pooled libraries for subsequent sequencing on Illumina systems. The goal of this user guide is to provide information and instructions for users to achieve high-quality sequence data from samples. This sequencing data is the input for the subsequent metagenomics data analysis (a separate user guide is provided). The methods described in this user guide may be applied to respiratory and other sample types.

1.2 The Workflow of RPIP powered by Explify

Table 1 provides an overview of the major activities in the workflow of RPIP powered by Explify in the wet bench environment (Pre-Amplification and Post-Amplification labs) for up to 18 samples including 2 external controls. Time estimates are included for planning purposes.

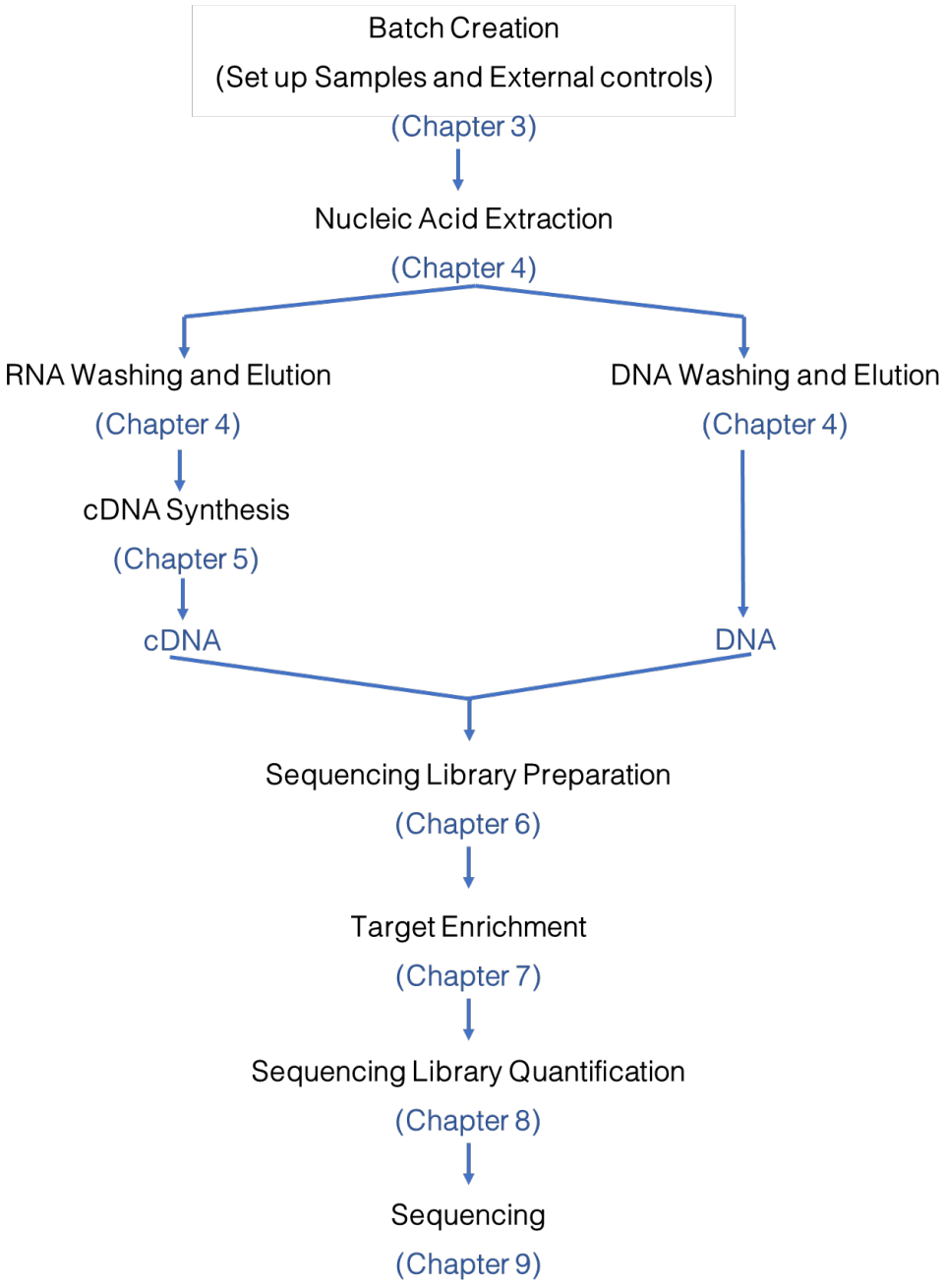
Table 1. Overview of the workflow of RPIP powered by Explify

Activity	Hands-on Time (hours)	Total Time (hours)
Batch Building	0.5	0.5
Extraction	2	2.5
cDNA Synthesis	1	3
Library Preparation	1.5	2.5
Enrichment	2	5
Library Quantification	0.5	2
Pooling and Loading	0.5	0.5
MiniSeq System (High-Output Kit)	-	10
Total	**8**	**26***

* The workflow could be further reduced to less than 24 hours using the MiniSeq Rapid Kit.

Figure 1 on the next page provides additional details for the wet bench activities.

Figure 1. Example workflow of RPIP



2. Equipment, Reagents and Consumables

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Equipment, Reagents, and Consumables

2.1 Equipment

The Explify workflow has been optimized using the specific equipment listed in Table 2.

Table 2. Equipment List

Location	Equipment	Recommendations	Procedure
Pre-Amplification Lab	Biosafety Cabinet	Appropriate for handling of potentially infectious substances	RNA/DNA extraction
	Small Tabletop Centrifuge	Able to accommodate 1.8-2.0 mL centrifuge tubes	RNA/DNA extraction
	Vortex with tube holder	Vortex Genie 2 or equivalent	RNA extraction
	Horizontal Microtube Holder for 24 Tubes	Compatible with the vortex	RNA extraction
	Vortex with normal cap	Vortex Genie 2 or equivalent	RNA/DNA extraction, cDNA synthesis, Library prep
	PCR workstation	Optimal protection from sample contamination (ThermoFisher model CBS Scientific P03002, recommended)	cDNA synthesis, Library prep
	IsoFreeze PCR Tube Chiller	Able to maintain sample temperature of less than 4 °C at room temperature	cDNA synthesis, Library Prep
	Microcentrifuge fitted for strip tubes	Able to accommodate PCR tube strips	cDNA synthesis, Library prep
	Microcentrifuge fitted for tubes	Able to accommodate 1.8 mL Eppendorf tubes or equivalent	cDNA synthesis, Library prep
	Magnetic Stands	Able to accommodate PCR tube strips	cDNA synthesis, Library prep
	Thermocycler	ThermoFisher ProFlex recommended	cDNA synthesis, Library prep
	Pipette-2 µL	For volumes from 0.5 µL to 2 µL	cDNA synthesis
	8-channel Pipette-20 µL	For volumes from 2 µL to 20 µL	cDNA synthesis, Library prep
	8-channel Pipette-200 µL	For volumes from 20 µL to 200 µL	cDNA synthesis, Library prep
	Pipette-20 µL	For volumes from 2 µL to 20 µL	RNA/DNA extraction, cDNA synthesis
	Pipette-200 µL	For volumes from 20 µL to 200 µL	RNA/DNA extraction, cDNA synthesis, Library prep
	Pipette-1000 µL	For volumes from 200 µL to 1000 µL	RNA/DNA extraction
	8-channel adjustable Pipette-1200 µL	For volumes up to 1200 µL, with adjustable spacing	RNA/DNA extraction

Location	Equipment	Recommendations	Procedure
Post-Amplification Lab	Vortex with normal cap	Vortex Genie 2 or equivalent	Quantification, Sequencing
	Microcentrifuge fitted for strip tubes	Able to accommodate PCR tube strips	Quantification, Sequencing
	Microcentrifuge fitted for tubes	Able to accommodate 1.8 mL Eppendorf tubes or equivalent	Quantification, Sequencing
	PCR workstation	Optimal protection from sample contamination (ThermoFisher model CBS Scientific P03002, recommended)	Library prep, Target Enrichment, Quantification
	Thermocycler	ThermoFisher ProFlex recommended	Library prep (PCR)
	Magnetic Stands	Able to accommodate PCR tube strips	Library prep
	Heat block	Able to accommodate 2mL microtubes and a Temperature set up to 60°C	Target Enrichment
	Pipette-20 µL	For volumes from 2 µL to 20 µL	Quantification, Sequencing
	Pipette-200 µL	For volumes from 20 µL to 200 µL	Library prep, Quantification, Sequencing
	Pipette-1000 µL	For volumes from 200 µL to 1000 µL	Library prep, Quantification, Sequencing
	8-channel Pipette-20 µL	For volumes from 2 µL to 20 µL	Library prep, Quantification
	8-channel Pipette-200 µL	For volumes from 20 µL to 200 µL	Library prep, Quantification
	Plate Centrifuge	Able to accommodate 96-well plates	Library prep, Quantification
	Quantitative PCR (qPCR)	ThermoFisher QuantStudio 3 (0.2 mL well model) recommended	Quantification
	Sequencer	Illumina MiniSeq recommended	Sequencing

2.2 Reagents

The reagents listed in Table 3 are necessary when following this user guide.

Table 3. Reagents List

Activity	Reagent	Supplier	Catalogue No.	Storage Conditions
Extraction	2x Concentrate DNA/RNA Shield	Zymo Research	R1200-125	Room temperature
	ZR Bashing Beads (0.1 & 0.5 mm)	Zymo Research	S6012-50	Room temperature
	Spin Away Filter (yellow column)	Zymo Research	C1006	Room temperature
	DNA/RNA Lysis Buffer	Zymo Research	D7001-1	Room temperature
RNA extraction	Ethanol	Sigma-Aldrich	E7023-500ML	Room temperature
	Zymo Spin IIC columns (Clean & Conc. 25)	Zymo Research	C1011	Room temperature
	RNA Prep Buffer	Zymo Research	R1060-2	Room temperature
	RNA Wash Buffer	Zymo Research	R1003-3	Room temperature
	DNase I Enzyme	Zymo Research	E1009-A	Room temperature, frozen at -20 °C after reconstitution
	DNase I Digestion buffer	Zymo Research	E1010-1-1	Room temperature
DNA extraction	DNA/RNA Prep Buffer	Zymo Research	D7010-2	Room temperature
	DNA/RNA Wash Buffer	Zymo Research	D7010-3	Room temperature
Library prep and Enrichment	Ethanol	Sigma-Aldrich	E7023-500ML	Room temperature
	Respiratory Pathogen ID/AMR Enrichment Kit Set A or Set B (RUO) (96 indexes, 96 samples)	Illumina	20047050 or 20046969	Room temperature, 2-8 °C, and -20 °C
	AMPure XP beads	Beckman Coulter	A63881	2-8 °C
Quantification	KAPA DNA Library Quantification Kit	Roche	KK4824	-20 °C
	1M Tris-HCl pH8.0	Invitrogen	15568-025	Room temperature
	Tween-20	Sigma-Aldrich	11332465001	Room temperature

Activity	Reagent	Supplier	Catalogue No.	Storage Conditions
Sequencing	MiniSeq High Output Sequencing Reagent (150 cycles)	Illumina	FC-420-1002	2-8 °C and -20 °C
	PhiX Control V3 kit	Illumina	FC-110-3001	-20 °C
	10N Sodium Hydroxide (NaOH)	Sigma-Aldrich	72068-100mL	Room temperature
	1M Tris-HCl pH7.0	Thermo Fisher	AM9851	Room temperature
Sample pre-treatment	Saline (0.85%)	Hardy Diagnostics	U155	Room temperature
	Sputo-LR	G Biosciences	786-1086	Room temperature
General	Molecular Biology Grade (MBG) Water	Corning	46-000-CM	Room temperature
General cleaning/ disinfect	Sodium hypochlorite solution (Bleach)	Sigma-Aldrich	425044-250ML	Room temperature
	RNase Away	Molecular BioProducts	7002	Room temperature
	Isopropanol (70%)	Sigma-Aldrich	I9516-500ML	Room temperature
	Disinfectant	VWR	14212-963	Room temperature

2.3 Consumables

The consumables recommended for use with this user guide for Explify RPIP are listed in Table 4.

Table 4. Consumables List

Activity	Item	Supplier	Cat #	Note
RNA extraction	Collection tube	Zymo Research	C1001	
	2.0 mL microcentrifuge tubes (sterile, DNase/RNase free)	VWR Scientific	20901-540	
	15 mL Conical tube (sterile)	VWR Scientific	62406-200	
cDNA synthesis	15 mL Conical tube (sterile)	VWR Scientific	62406-200	
	8-Tube Strip, without Caps, 0.2mL (sterile, DNase/RNase free)	VWR Scientific	20170-002	
	Bubble Cap Strip (Caps only)	VWR Scientific	20170-000	need to match the tube strip
DNA extraction	Collection tube	Zymo Research	C1001	
	15 mL Conical tube (sterile)	VWR Scientific	62406-200	
Library prep	15 mL Conical tube (sterile)	VWR Scientific	62406-200	
	2.0 mL microcentrifuge tubes (sterile, DNase/RNase free)	VWR Scientific	20901-540	
	8-Tube Strip, without Caps, 0.2mL (sterile, DNase/RNase free)	VWR Scientific	20170-002	
	Bubble Cap Strip (Caps only)	VWR Scientific	20170-000	need to match the tube strip
Quantification	8-Tube Strip, without Caps, 0.2mL (sterile, DNase/RNase free)	VWR Scientific	20170-002	
	Bubble Cap Strip (Caps only)	VWR Scientific	20170-000	need to match the tube strip
	2.0 mL microcentrifuge tubes (sterile, DNase/RNase free)	VWR Scientific	20901-540	
	Axygen Prebarcoded PCR Plates	VWR Scientific	10032-806	need to match the qPCR instrument
	PCR Plates	VWR Scientific	14005-710	serial dilution
	MicroAmp™ Optical Adhesive Film	Thermo Fisher	4311971	need to match the qPCR instrument/plate
	Square PET Media Bottles, 250 mL (Sterile)	VWR Scientific	76302-836	
Sample pretreatment	Pipette tips P-1000 wide Bore (filtered, sterile)	Rainin	30389218	
General	1.5 mL microcentrifuge tubes (sterile, DNase/RNase free)	VWR Scientific	20170-650	
	PVC Reservoirs (sterile)	VWR	89094-688	
	Pipette tips P-20 (filtered, sterile)	Rainin	30389225	

Activity	Item	Supplier	Cat #	Note
General	Pipette tips P-200 (filtered, sterile)	Rainin	30389239	
	Pipette tips P-1000 (filtered, sterile)	Rainin	30389212	
	1.5 mL DNA LoBind tubes	Eppendorf	22431021	

3. Batch Creation Procedure

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Batch Creation Procedure

3.1 Components of a Batch

- Samples to test

NOTE: Collect sample information, including sample identifier(s) and sample type(s).

- Two external controls—one negative control and one positive control (See Appendix A for control recommendation)

NOTE: External controls must be included in each batch and processed together withthe test samples throughout the workflow.

- Blank control(s), is recommended to detect contamination introduced during the experiment.

NOTE: In cases in which one or more of the samples in the batch is diluted because of insufficient volume or high viscosity (see Appendix B for high viscosity sample treatment), the reagent used to dilute the sample, e.g., water or saline, is used as the blank control.

3.2 Decisions for Batch Building

1. The type of sequencing kit determines the number of samples that can fit in a sequencing run.
2. When more than one positive control is available, alternate the positive controls between consecutive runs.
3. Inspect each sample for volume and viscosity. If the sample is highly viscous, pre-treat the sample follow the procedure listed in Appendix B. If the sample has insufficient volume, increase the sample volume by adding molecular biology grade (MBG) water. In either case, use it as blank control.
4. Arrange the sample(s) and controls to assign a Well Identification (Well ID) number to each of them. This number will be used throughout the process to associate the intermediate steps with the primary sample(s) and controls.

4. DNA/RNA Extraction Procedure

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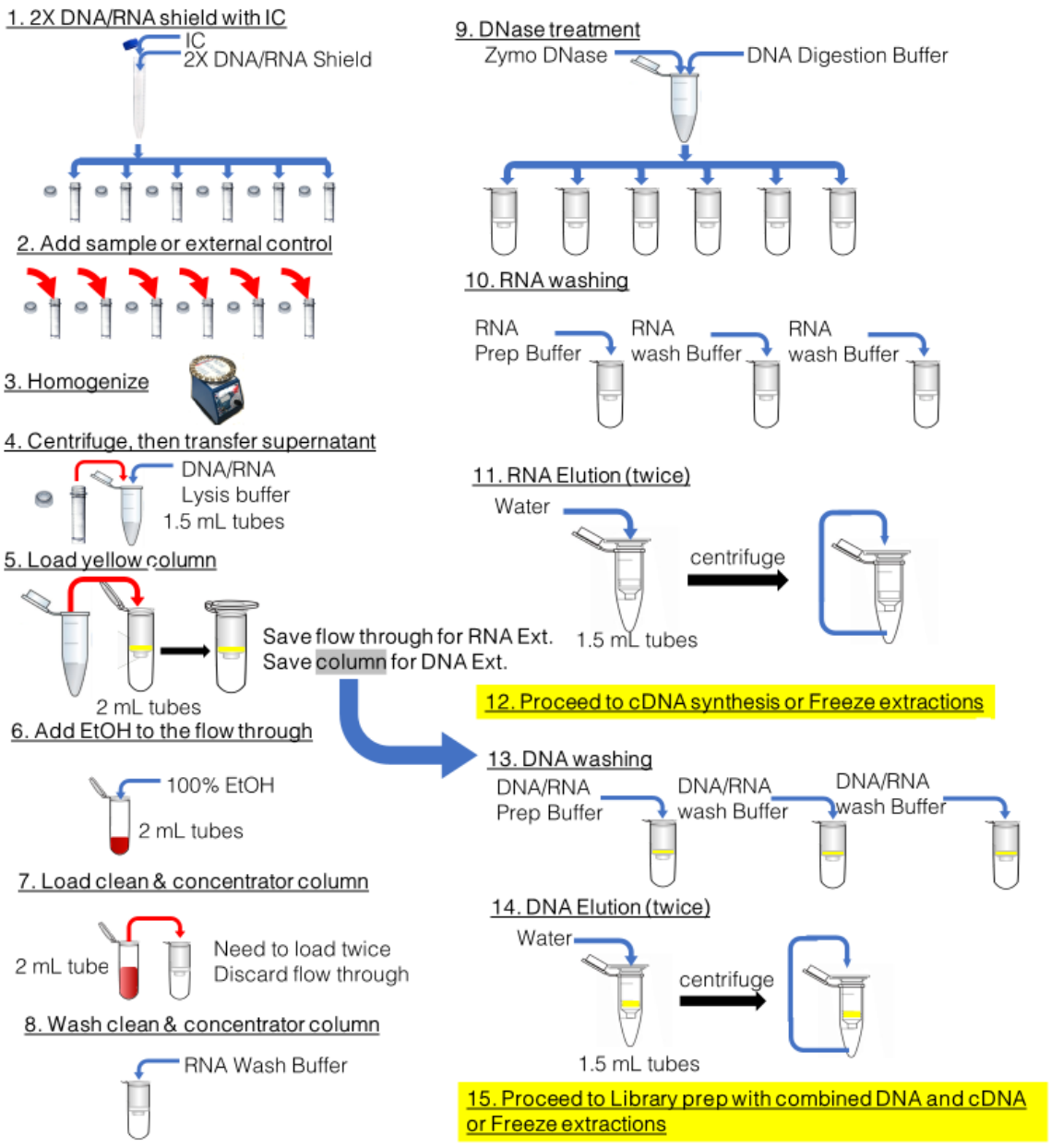
DNA/RNA Extraction Procedure

DNA and RNA from samples can be extracted using the procedure shown in Figure 2.

Figure 2: Overview of DNA/RNA Extraction Procedure



After extraction, the purified RNA can be used to make cDNA immediately or stored at -80 °C for up to one month.

IMPORTANT All steps of the procedure must be performed in a Pre-amplification Lab.

4.1 Extraction Setup

1. Clean the biosafety cabinet, benchtop, and pipettes, first with disinfectant, then with 70% Isopropanol, then with RNase Away.
2. Aliquot 10 mL of MBG water into a 15 mL conical tube for use throughout the entire extraction procedure.
3. Prepare the tubes shown in table 5 for each sample. Label each tube with the correct Well ID (Chapter 3).

Table 5. Setup of the Extraction for each sample

Tubes	Quantity
Bashing Bead Tube (Zymo S6012-50)	1
1.5 mL microcentrifuge tube	3
Spin Away Filter (yellow column) (Zymo C1006)	1
2.0 mL microcentrifuge tube	1
Zymo-Clean and Concentrator-25 Column (white column) (Zymo C1011)	1
Open-top Collection tubes (Zymo C1001)	9

4.2 Sample Homogenization and Cell Lysis

IMPORTANT Aseptic technique must be applied. All sample transfer steps should be performed in a biosafety cabinet.

1. Retrieve all sample(s) and external controls (see Appendix A) to be tested and thawed (if frozen). Briefly vortex and centrifuge, then place on ice.

NOTE: All samples and/or external controls will be referred to as “samples” from this point.

2. Retrieve the selected Internal Controls from the -80°C refrigerator and thaw. Briefly vortex and centrifuge, then place on ice.

NOTE: Internal controls can be selected from the recommended list (Appendix A). T7 is preferred as internal control.

3. In a new tube, prepare an adequate volume of Extraction Reagent Mix for the number of samples in the batch (see Table 6). Add one dead volume of mixture when preparing the mix.

Table 6. Extraction Reagent Mix Preparation

	# of samples	
Reagent	1	18*
Internal Control (µL)	2	38
2x DNA/RNA Shield (µL) (Zymo R1200-125)	400	7600
Total Volume (µL)	402	7638

*One dead volume included.

4. Add 402 µL of Extraction Reagent Mix into each empty Bashing Bead Tube.
5. In the biosafety cabinet, add 400 µL of sample into the corresponding BashingBead Tube. Secure all sample tubes onto a tabletop vortex Genie 2, fitted with a Horizontal Microtube Holder for 24 Tubes (VWR Cat#SI-H524).
6. Homogenize samples by vortexing for 10 minutes at maximum speed.
7. Centrifuge the samples at 10,000 rcf for 2 minutes on a small tabletop Eppendorf centrifuge.
8. Retrieve one labeled 1.5 mL tube for each sample.
9. Add 400 µL of DNA/RNA Lysis Buffer (room temperature, Zymo D7001-1) each to the labeled tubes.
10. In the biosafety cabinet, transfer 400 µL of sample homogenate supernatant from step 7 to the tube containing DNA/RNA Lysis Buffer. Mix thoroughly by vortexing for 5 seconds on a Genie 2 fitted with a regular plastic cap. The lysate is ready for extraction.
11. Continue immediately to next section.

4.3 DNA Binding

1. Retrieve the labeled Spin-Away Filters (Yellow Column) for all samples being tested.
2. Transfer 800 µL of the samples from Step 4.2 into a Spin-Away Filter (Yellow Column) in a 2 mL labeled tube and centrifuge at 10,000 rcf for 30 seconds on a small tabletop centrifuge.
3. Place the columns into fresh collection tubes and save for later steps of DNA washing and elution.
4. Continue with RNA purification from the flow-through in the 2 mL tube.

4.4 RNA Binding

1. Retrieve one Zymo-Spin IIC (Clean and Concentrator-25) Column in a collection tube per sample, label each column on the lid.
2. Add 800 µL of 100% Ethanol to the 2 mL tube containing RNA flow-through and mix well by vortexing for 5 seconds on a vortex Genie 2 fitted with a normal plastic cap.
3. Transfer 800 µL of the mixture to a Zymo-Spin IIC (Clean and Concentrator-25) Column and centrifuge at 10,000 rcf for 30 seconds on a small tabletop centrifuge.

4. Replace the collection tube containing the flow-through with a new collection tube for each column. Discard the old collection tube and the flow-through.
5. Repeat steps 3-4 until all volume passes through the Zymo-Spin IIC (Clean and Concentrator-25) Column.

NOTE: If the Zymo-Spin Clean and Concentrator-25 Column becomes clogged, centrifuge the sample at 16,000 rcf for 10 minutes. Discard any volume that does notrun through the column and proceed to step 6.

NOTE: Zymo RNA Wash Buffer must be reconstituted with 100% Ethanol. Follow the manufacturer's instructions and be sure to check the box on the bottle after reconstitution.

6. Add 400 µL of RNA Wash Buffer (Zymo R1003-3) to the Zymo-Spin IIC (Clean and Concentrator-25) Column and centrifuge at 10,000 rcf for 30 seconds on a small tabletop centrifuge.
7. For each column, replace the collection tube containing the flow-through with a new collection tube. Discard the old collection tube and the flow-through.
8. Continue immediately to next section.

4.5 DNase Treatment of RNA

1. Retrieve the Zymo DNase I from the -20 °C freezer to thaw. Briefly centrifuge and place on ice.

NOTE: Zymo DNase I arrives as lyophilized powder. Follow the manufacturer's instructions to reconstitute the enzyme with MBG water. Aliquot the reconstituted enzyme and freeze the aliquots at -20 °C. Do not repeatedly freeze-thaw the reconstituted enzyme.

2. Retrieve DNA Digestion Buffer from room temperature. Briefly vortex.
3. In a separate new tube, prepare the DNase Reagent Mix according to the number oftesting samples, with one dead volume as shown in Table 7.

Table 7. DNase Reagent Mix Preparation

	# of samples	
Reagent	1	18*
Zymo DNase I (µL)	5	95
Zymo DNA Digestion Buffer (µL)	75	1425
Total Volume (µL)	80	1520

*One dead volume included.

4. Add 80 µL of the DNase Reagent Mix directly to the Zymo-Spin IIC (Clean and Concentrator-25) Column matrix.
5. Incubate at room temperature for 15 minutes.
6. Continue immediately to next section.

4.6 RNA Washing and Elution

NOTE: Use an adjustable multichannel P-1200 pipette (optional) for the wash steps andchange tips after each use.

1. Add 400 µL of RNA Prep Buffer (Zymo R1060-2) to the column and centrifuge it at 10,000 rcf for 30 seconds on a small tabletop Eppendorf centrifuge.
2. Replace the collection tube containing the flow-through with a new collection tube for each column. Discard the old collection tube and the flow-through.
3. Add 700 µL of RNA Wash Buffer (reconstituted) to the column and centrifuge it at 10,000 rcf for 30 seconds.
4. Replace the collection tube containing the flow-through with a new collection tube for each column. Discard the old collection tube and the flow-through.
5. Add 400 µL of RNA Wash Buffer to the column and centrifuge it at 10,000 rcf for at least **2 minutes** to ensure complete removal of the wash buffer.
6. Retrieve one 1.5 mL, labeled elution tube for each sample.
7. Leave the collection tube in the centrifuge to be discarded later. Carefully remove the column from each sample tube and place into the new 1.5 mL microcentrifuge tube for elution.

IMPORTANT Do not splash or disrupt the flow-through in the collection tube.

8. Add 20 µL of MBG water directly to the Zymo-Spin Clean and Concentrator-25 Column matrix.
9. Let the columns stand at room temperature for 5 minutes.
10. Centrifuge at 8,000 rcf on a small tabletop Eppendorf centrifuge for 1 minute to elute RNA from the Zymo-Spin Clean and Concentrator-25 Columns.
11. Re-apply the flow-through elution water from previous step (up to 20 µL) directly to the Zymo-Spin Clean and Concentrator-25 Column matrix again and let it stand at room temperature for another 5 minutes.

12. Centrifuge at 8,000 rcf for 1 minute to elute additional RNA from the Zymo-Spin Clean and Concentrator-25 Column.
13. Proceed to cDNA synthesis or store the RNA at -80 °C for up to one month.

4.7 DNA Washing and Elution

NOTE: The following steps can be done during the 15 minutes DNase incubation for RNA extraction or incubation time during cDNA synthesis.

1. Add 400 µL of DNA/RNA Prep Buffer (Zymo D7010-2) to each of the saved Spin-Away Filter (Yellow column) and centrifuge it at 10,000 rcf for 30 seconds.
2. Replace the collection tube containing the flow-through with a new collection tube for each column. Discard the old collection tube and the flow-through.
3. Add 700 µL of DNA/RNA Wash Buffer to the column and centrifuge it at 10,000 rcf for 30 seconds.

NOTE: Zymo DNA/RNA Wash Buffer must be reconstituted with 100% Ethanol. Follow the manufacturer's instructions. Check the box on the bottle after reconstitution.

4. Replace the collection tube containing the flow-through with a new collection tube for each column. Discard the old collection tube and the flow-through.
5. Add 400 µL of DNA/RNA Wash Buffer to the column and centrifuge it at 10,000 rcf for at least 2 minutes to ensure complete removal of the wash buffer.
   Retrieve the labeled 1.5 mL elution tubes for all samples being tested (from Section 6.1 Extraction Setup).
6. Leaving the collection tube in the centrifuge, carefully remove the column from each sample tube and place into the labeled 1.5 mL elution tube. Be careful to avoid splashing the sample or disrupting the flow-through in the original collection tube.
7. Add 50 µL of MBG water directly to the column matrix and let the column(s) stand for 5 minutes.
8. Centrifuge at 8,000 rcf for 1 minute to elute DNA from the column.
9. Re-apply the flow-through elution water from previous step (up to 50 µL) to the column matrix and let it stand at room temperature for 5 minutes.
10. Centrifuge at 8,000 rcf for 1 minute to elute additional DNA from the column.

SAFE STOPPING POINT: Store the extracted nucleic acid frozen at -80 °C for up to 6 months.

5. cDNA Synthesis Procedure

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cDNA Synthesis Procedure

NOTE: It is recommended that library preparation and all following steps be performed inside of a PCR workstation to prevent possible contamination.

NOTE: The input material for this procedure is extracted RNA from the RNA Extraction Procedure. The **Respiratory Pathogen ID/AMR Enrichment Kit** will be used for the rest of the protocol.

NOTE: PCR strip tubes must be securely capped during all centrifugation, vortexing and incubation steps. Do not reuse the caps. Leave the caps off during air dry steps.

5.1 Denature RNA

IMPORTANT This step must be performed in the Pre-Amplification Lab.

1. Thaw Elute, Prime, Fragment High Concentration Mix (EPH3) included in the Respiratory Pathogen ID/AMR Enrichment Kit at room temperature, vortex and quick spin; thaw First Strand Synthesis Act D Mix (FSA) included in the Respiratory Pathogen ID/AMR Enrichment Kit on ice, invert to mix and centrifuge briefly; keep on ice after thawing.
2. Retrieve and label a new set of 0.2 mL 8-tube PCR strip(s). Place the labeled tube strip(s) on PCR tube chiller.
3. On the PCR tube chiller, add 8.5 µL of EPH3 to each tube of the PCR strip(s).
4. Add 8.5 µL of extracted RNA from each sample to the corresponding tube, then pipette 10 times to mix, centrifuge briefly.
5. Start the Primer Annealing Thermocycler Program (see Table 8) on thermocycler, then set the instrument on 'Pause' at the first step (65 °C).
6. Place the tube strip(s) into the thermocycler, then resume the program.

Table 8. Primer Annealing Thermocycler Program

Step	Temp (°C)	Time	Cycle #
1: Primer annealing	65	5 mins	1
2: Final hold	4	∞	1

Total time: 5 minutes. Cover temperature: 100 °C.

5.2 First Strand Synthesis

1. While the Primer Annealing Program is in progress, retrieve and label a new set of 0.2 mL 8-tube PCR strip(s). Place the labeled tube strip(s) on a chilled PCR tube chiller.
2. Retrieve Resuspension Buffer (RSB) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer, and thaw at room temperature. Vortex and centrifuge briefly then place on ice. Retrieve Second Strand Marking Master Mix (SMM) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer, thaw on ice. Invert to mix and then centrifuge briefly.

3. Retrieve Reverse Transcriptase (RVT) included in the Respiratory Pathogen ID/AMR Enrichment Kit when needed. Flick to mix, and then centrifuge briefly. Put back immediately for storage after using.
4. Prepare the First Strand Synthesis Reagent Mix in a new tube according to the number of samples, adding 1 additional dead volume as shown in Table 9. Mix the Reagent Mix by pipetting or flicking, then centrifuge briefly, keep on ice.

IMPORTANT Do not vortex First Strand Synthesis Reagent Mix.

Table 9. First Strand Synthesis Reagent Mix Preparation

	# of samples	
Reagent	1	18*
FSA (µL)	7.2	136.8
RVT (µL)	0.8	15.2
Total Volume (µL)	8	152

*One dead volume included.

5. After the Primer Annealing Program reaches the final hold temperature, remove the tube strip(s) and centrifuge briefly. Place the tube strip(s) on a chilled PCR tube chiller.
6. Add 8 µL of First Strand Synthesis Reagent Mix to each sample. Pipette to mix and centrifuge briefly.
7. Start the First Strand Synthesis Thermocycler Program (see Table 10) on a thermocycler. Set the instrument on Pause once the thermocycler reaches the temperature of the first step (25 °C).
8. While the instrument is on Pause, place the tube strip(s) into the thermocycler and resume the program.

Table 10. First Strand Synthesis Thermocycler Program

Step	Temp (°C)	Time	Cycle #
1: First strand synthesis	25	10 mins	1
2: First strand synthesis, cont'd	42	15 mins	1
3: Enzyme inactivation	70	15 mins	1
4: Final hold	4	∞	1

Total time: ~40 minutes. Cover temperature: 100°C.

9. Once the First Strand Synthesis Program reaches the final hold temperature, remove the tube strip(s) from the thermocycler; centrifuge briefly, and then place it on a chilled PCR tube chiller.
10. Continue immediately to next section.

5.3 Second Strand Synthesis

IMPORTANT This procedure must be performed in Pre-Amplification Lab.

1. Start the Second Strand Synthesis Thermocycler Program (see Table 11) on thermocycler and set the instrument on Pause at the first step (16 °C).

Table 11. Second Strand Synthesis Thermocycler Program

Step	Temp (°C)	Time	Cycle #
Second strand synthesis	16	60 min	1
Final hold	4	∞	1

Total time: ~60 minutes. Cover temperature: 40°C.

2. Retrieve AMPure XP beads from the 2-8 °C refrigerator in the Pre-Amplification Lab and leave at room temperature for at least **30 minutes** in preparation for Section 5.4.
3. Invert thawed SMM to mix, then centrifuge briefly.
4. On the chilled PCR tube chiller, carefully removed the strip caps and discard. Add 25 µL of SMM to each sample, pipette 10 times to mix. Close the tubes with new set of caps and centrifuge briefly.
5. While the instrument is on Pause, place the tube strip(s) into the thermocycler and resume the program.
6. Once the Second Strand Synthesis Program reaches the final hold temperature, remove the tube strip(s) from thermocycler, centrifuge briefly, then place it on a rack at room temperature.
7. Continue immediately to next section.

5.4 cDNA Purification

IMPORTANT This procedure must be performed in Pre-Amplification Lab.

1. Prepare a fresh solution of 80% ethanol using 8 parts of 100% ethanol and 2 parts of MBG water.
2. Ensure that the AMPure XP beads have been at room temperature for at least 30 minutes. Resuspend the beads by vortexing the tube for 10 seconds.
3. Add 90 µL of AMPure XP beads suspension to each sample tube from the Second Strand Synthesis step and mix by vortexing for 5 seconds.

NOTE: Change pipette tip after each pipetting step to prevent cross-contamination.

4. Incubate all samples at room temperature for 5 minutes.
5. Centrifuge tube strip(s) briefly and transfer tube strip(s) to a magnetic stand and allow it to stand for 1 minute while the beads settle.
6. Keep the tube strip(s) in the magnetic stand and use a multi-channel P-200 pipette to remove and discard the buffer from each tube.

NOTE: Do not disrupt beads bound to the side of the tube while pipetting.

7. Keep the tube strip(s) in the magnetic stand, and with a multi-channel P-200 pipette add 175 µL of 80% ethanol to each tube. Note that the beads should remain bound to the side of the tube.
8. Let the tube strip(s) stand for at least 30 seconds and then remove all the 80% ethanol wash from each tube with a multi-channel P-200 pipette. Do not disperse the beads.The tube strip(s) should remain firmly in the magnetic stand throughout the process.
9. Repeat the ethanol wash (steps 7-8) for a total of 2 washes.
10. After removal of the second wash, collect residual ethanol to the bottom of the tubes by centrifuging for 2 seconds; place the tube strip(s) back on the magnetic stand,and then pipette to remove all residual ethanol with a multi-channel P-20 pipette.
11. Air dry the tube strip(s) at room temperature for 2 minutes on the magnetic stand.
12. Inspect each tube to ensure that ethanol has completely evaporated. If there is residual ethanol, let the tubes air dry until there is no visible ethanol.
13. Vortex RSB for 5 seconds immediately before use. Remove the tube strip(s) from the magnetic stand and add 17 µL of RSB to each tube. Vortex to mix.
14. Let tubes stand at room temperature for 2 minutes then centrifuge briefly.
15. Place the tube strip(s) on a magnetic stand for 1 minute.
16. Transfer 15 µL of the clear supernatant from each sample on the magnetic stand to a new set of labeled tube strip(s). Label this set of tube strip(s) **cDNA.**

SAFE STOPPING POINT: Store the cDNA frozen at -20 °C for up to 7 days.

6. Sequencing Library Preparation Procedure

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Sequencing Library Preparation Procedure

NOTE: PCR strip tubes must be securely capped during all centrifugation, vortexing, andincubation steps. Do not reuse the caps. Leave the caps off during air dry steps.

6.1 Combination of cDNA and DNA

IMPORTANT This procedure must be performed in the Pre-Amplification Lab.

1. Retrieve extracted DNA and thaw if necessary, vortex and centrifuge briefly.
2. Add 15 µL of DNA directly into the corresponding cDNA strip tube, vortex and centrifuge briefly. Save the rest of DNA at -80 °C for up to 6 months.
3. Use the combined material for the following steps.

6.2 Tagmentation

IMPORTANT This procedure can be performed in the Pre-Amplification Lab.

1. Retrieve Bead-Linked Transposomes (EBLTL) included in the Respiratory Pathogen ID/AMR Enrichment Kit at 2-8 °C refrigerator, allow it warm to room temperature for 20 mins. Vortex until beads are resuspended prior to use.
2. Retrieve Tagmentation Buffer (TB1) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer and thaw at room temperature. Vortex to mix and centrifuge briefly.
3. Retrieve the index adapter (UDI) plate included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer and thaw at room temperature.
4. Prepare the Tagmentation Reagent Mix in a separate new 1.5 mL tube according to the number of libraries, as shown in Table 12. Add one dead volume to the mixture for pipetting errors.

Table 12. Tagmentation Reagent Mix Preparation

	# of samples	
Reagent	1	18*
Bead-Linked Transposomes (EBLTL) (µL)	10	190
Tagmentation Buffer (TB1) (µL)	10	190
Total Volume (µL)	20	380

*One dead volume included.

5. Vortex the Tagmentation Reagent Mix for 10 seconds and visually inspect the tubeto ensure the beads are evenly resuspended.

6. Add 20 µL of the Tagmentation Reagent Mix to each well of the 0.2 mL PCR strip(s) with combined cDNA and DNA (from Section 6.1).
   Place the tubes into thermocycler and start the Tagmentation Program (seeTable 13).

Table 13. Tagmentation Program

Step	Temp (°C)	Time	Cycle #
1: Tagmentation	55	5 min	1
2: Final hold	10	∞	1

Total time: ~5 minutes. Cover temperature: 100 °C.

7. After the program reaches the final hold temperature, remove the tube strip(s) from thermocycler and centrifuge briefly, then let stay at room temperature for 2 minutes.
8. Continue immediately to next section.

6.3 Stop Tagmentation

IMPORTANT This procedure can be performed in the Pre-Amplification Lab.

1. Retrieve Stop Tagment Buffer 2 (ST2) included in the Respiratory Pathogen ID/AMR Enrichment Kit from room temperature, vortex and centrifuge briefly.
2. Add 10 µL of ST2 to each sample tube (section 6.2). Seal the strip tubes and vortex for 5 seconds and then centrifuge briefly.
3. Incubate the samples at room temperature for 5 minutes.
4. After the incubation time, centrifuge the samples briefly.

6.4 Post-Tagmentation Purification

IMPORTANT This procedure can be performed in the Pre-Amplification Lab.

1. Place the strip tubes on a magnetic stand and wait until the liquid is clear.
2. Retrieve the Tagment Wash Buffer (TWB) included in the Respiratory Pathogen ID/AMR Enrichment Kit and vortex briefly.

IMPORTANT In the following steps 3-10, proceed ‘strip-by-strip’ to avoid drying out of the beads: remove the supernatant from one tube strip, immediately add TWB and mix the samples in the same tube strip, then proceed with the next tube strip.

3. Carefully remove and discard all supernatant (~60 µL) from the strip tube, ensuring not to disrupt the beads on the side of the tube.
4. Remove the tube strip from the magnetic stand.
5. Add 100 µL of TWB to each library tube and mix by pipetting until beads are resuspended completely.
6. Place the tubes onto a magnetic stand for 2 minutes.
7. Carefully remove and discard all supernatant (~100 µL), ensuring not to disrupt the beads on the side of the tube.
8. Remove the tube from the magnetic stand
9. Repeat the TWB wash (steps 5 through 8) twice for a total of 3 washes.
10. After the final wash, place the tubes onto a magnetic stand for at least 2 minutes. The tubes can stay on the magnetic rack in TWB until the PCR Master Mix is ready (see the following section).
11. Continue immediately to next section.

6.5 PCR Amplification of Libraries

IMPORTANT This procedure must be performed in the Post-Amplification Lab.

1. Retrieve the Enhanced PCR Mix (EPM) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer and thaw in a 2-8 °C refrigerator or on ice. Invert the reagent tube 5 times to mix; centrifuge briefly, then place on ice.
2. Prepare the PCR Master Mix in a separate new tube according to the number of libraries as shown in Table 14. Add one dead volume to the mixture to account for pipetting errors.

Table 14. PCR Master Mix Preparation

	# of samples	
Reagent	1	18*
Enhanced PCR Mix (EPM) (µL)	20	380
MBG Water (µL)	20	380
Total Volume (µL)	40	760

*One dead volume added.

3. Vortex the PCR Master Mix for 5 seconds and centrifuge briefly.
4. Optional, aliquot the PCR Master Mix into an 8-well strip tube in order to quickly add it to the samples and to avoid drying the beads.

IMPORTANT In the following steps 5-6, proceed ‘strip-by-strip’ to avoid drying out of the beads. Remove the supernatant from one tube strip, immediately add the PCR master mix to the same tube strip, then proceed to the next tube strip.

5. Carefully remove and discard all supernatant (~100 µL) from each sample on the magnetic stand from Section 6.4 above, ensuring not to disrupt the beads on the side of the tube. Use a P-20 pipette to remove any remaining TWB wash buffer at the bottom of the tube. However, residual foam in the tube should not inhibit the following PCR reaction. Proceed immediately to the next step to avoid over drying of the beads.
6. Remove tubes from the magnetic stand. Add 40 µL of PCR Master Mix to each sample tube. After all tubes received the Master Mix, pipette to mix 10 times or until the beads are thoroughly resuspended.
7. Ensure that the UDI plate has been at room temperature for at least 20 minutes. Centrifuge the plate briefly.

IMPORTANT In the following steps 8-9, proceed 'strip-by-strip' to avoid mistakes in adding indexes.

8. One tube strip at a time, with a P-20 multichannel pipette, use the pipette tips to pierce the cover of a set of wells on the index adapter plate, pipette 10 µL of the indexes from the plate, dispense into the library tube strip.

IMPORTANT UDI Indexes are for single use only in each batch of samples.

9. Record which adaptor wells were used for which sample and the UDI set identification.

IMPORTANT Always clearly record the index well added to each library. This information allows the correct association between sequencing data and libraries.

10. Pipette the sample 10 times to mix with a P-200 multichannel pipette set at 40 µL to avoid creating bubbles and seal the tube strip.
11. Centrifuge the tube strip(s) briefly, and place into a thermocycler in the Post-Amplification Lab and start Tagmentation PCR program (see Table 15).

Table 15. Tagmentation PCR Program

Step	Temp (°C)	Time	Cycle #
1: Initial hold	72	3 min	1
2: Initial denaturation	98	3 min	1
3: Denaturation	98	20 sec	17
4: Primer annealing	60	30 sec	
5: Extension	72	1 min	
6: Final extension	72	3 min	1
7: Final hold	10	∞	1

Total time: ~40 minutes. Cover temperature: 100 °C.

SAFE STOPPING POINT: Store the PCR products frozen at -20 °C for up to 7 days or leave on the thermocycler for up to 24 hours.

6.6 Post-PCR Purification

IMPORTANT This step of the procedure must be performed in Post-Amplification Lab.

NOTE: PCR strip tubes must be securely capped during all centrifugation, vortexing, and incubation steps. Do not reuse the caps. Leave the caps off during air dry steps.

1. Retrieve AMPure XP Beads and RSB from a 2-8 °C refrigerator and allow standing at room temperature for at least 30 minutes prior to using.
2. After the Tagmentation PCR programs reaches the final hold temperature, remove the tube strip(s) from thermocyclers and centrifuge briefly.
3. Place the tube strip(s) on a magnetic stand for 2 minutes to settle the beads.
4. When the liquid is clear, aspirate 45 µL of the PCR supernatant from tubes on magnetic stand (Step 3), transfer to the new set of strip tubes.
5. Ensure that AMPure XP Beads have been at room temperature for at least 30 minutes. Immediately before use, vortex briefly or until the beads are thoroughly resuspended.
6. Add 81 µL of AMPure XP Beads to the new strip tubes containing the amplified library from step 4. Cap the tubes and vortex briefly.
7. Let the tube strip stand at room temperature for 5 minutes, then centrifuge briefly.
8. Place the tube strip on a magnetic stand and let stand for 2 minutes.
9. Prepare a fresh solution of 80% ethanol using 8 parts of 100% ethanol and 2 parts of MBG water.

IMPORTANT In the following steps 10-11, proceed ‘strip-by-strip’ to avoid drying out of the beads.

10. After the incubation on the magnetic stand is complete, carefully remove and discard all supernatant (~120 µL) with a multi-channel P-200 pipette, ensuring not to disrupt the beads on the side of the tube.
11. Keeping the tube strip on the magnetic stand, add 200 µL of 80% ethanol with a multi-channel P-200 pipette and let stand for 30 seconds. Do not mix the solution.

IMPORTANT In the following steps 12-13, proceed ‘strip-by-strip’ to avoid drying out of the beads.

12. Remove and discard the ethanol.
13. Perform a second wash, by adding 200 µL of 80% ethanol and let stand for 30 seconds. Do not mix the solution.
14. Remove and discard the ethanol, cap the tube strip(s).
15. Remove the tube strip(s) from magnetic stand and centrifuge briefly.

16. Place the tube strip(s) onto magnetic stand, then use a multi-channel P-20 pipette to remove all residual ethanol from each sample tube.
17. Keep the tube strip(s) on the magnetic stand, let the tubes air dry for 2 minutes.
18. Vortex RSB briefly immediately before use.
19. Remove the tube strip from the magnetic stand and add 20 µL of RSB to each sample. Thoroughly mix each sample by pipetting or vortex briefly.
20. Let tubes stand at room temperature for 2 minutes then centrifuge briefly.
21. Place the tube strip(s) on a magnetic stand for 1 minute.
22. When the liquid is clear, transfer 18 µL of the clear supernatant from each sample on the magnetic stand to a set of new, labeled sample tube strip(s).

SAFE STOPPING POINT: Store the libraries frozen at -20 °C for up to 30 days.

7. Target Enrichment Procedure

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Target Enrichment Procedure

IMPORTANT This procedure must be performed in the Post-Amplification Lab.

NOTE: PCR strip tubes must be securely capped during all centrifugation, vortexing and incubation steps. Do not reuse the caps. Leave the caps off during air dry steps.

7.1 Pooling Pre-Enriched Libraries by Volume

1. This step combines every three libraries into one pool for three-plex enrichment.
2. Transfer 2.5 µL of each undiluted library to one well of a new strip tube for a total of three libraries per pool and a final volume of 7.5 µL.

NOTE: If pooling is not preferred, use 7.5 µL of a single library to perform the following procedure.

NOTE: If more than three libraries are pooled, use 2.5 µL each library to make the pool, concentrate the pool per Appendix C.

7.2 Hybridize Probes

1. Preheat the heat block to 50 °C.
2. Retrieve Enrichment Probe Panel from the -20 °C freezer and thaw at room temperature, then vortex briefly to mix.
3. Retrieve Enrich Hyb Buffer 2 (EHB2) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the 2-8 °C refrigerator and thaw at room temperature. Vortex to mix and ensure the solution is clear.
4. Retrieve Streptavidin Magnetic Beads (SMB3) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the 2-8 °C refrigerator and let stay at room temperature for at least 30 minutes prior to use.
5. Retrieve Hyb Buffer + IDT NXT Blockers (NHB2) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer and thaw at room temperature. Vortex at maximum speed 3 times for 10 seconds each, pipette to resuspend fully until the solution is clear, quick spin, keep warm at 50 °C until use.
6. Add the following reagents in the order listed to a new strip tube.
    - Pre-enriched library pool (7.5 µL)
    - NHB2 (12.5 µL)
    - Enrichment probe panel (2.5 µL)
    - EHB2 (2.5 µL)

7. Using a pipette set to 20 µL, pipette each well 10 times to mix, centrifuge briefly.
8. Place the tubes into thermocycler and start the Hybridization Program (see Table 17).
9. Preheat a second block on the thermocycler at 58 °C hold. Set the cover temperature at 70°C

Table 17. Hybridization Program

Step	Temp (°C)	Time	Cycle #
1	95	5 min	1
2	94	1 min	1
3	92	1 min	1
4	90	1 min	1
5	88	1 min	1
6	86	1 min	1
7	84	1 min	1
8	82	1 min	1
9	80	1 min	1
10	78	1 min	1
11	76	1 min	1
12	74	1 min	1
13	72	1 min	1
14	70	1 min	1
15	68	1 min	1
16	66	1 min	1
17	64	1 min	1
18	62	1 min	1
19	60	1 min	1
20*	58	90 min	1

Total time: ~120 minutes. Cover temperature: 100 °C.

* The minimum hybridization time at 58°C is 90 minutes as optimized by IDbyDNA. Extending the hold to up to 24 hours is optional for overnight hybridization. Without the extended hold, total program time is ~2 hours.

7.3 Capture Hybridized Probes

1. Ensure that SMB3 has stood at room temperature for at least 30 minutes prior to use.
2. Retrieve Enrichment Elution Buffer 1 (EE1), Enhanced Enrichment Wash (EEW) and 2 N NaOH (HP3) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer and thaw at room temperature, then vortex briefly to mix.
3. Prepare aliquot of EEW prior to use. Total 220 µL of EEW is needed for each reaction. Preheat aliquots of EEW on a 58 °C heat block.
4. Retrieve Elute Target Buffer 2 (ET2) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the 2-8 °C refrigerator and thaw at room temperature, vortex to mix.

5. Prepare the Elution Master Mix in a new tube according to the number of library pools, as shown in Table 18. Add 10% dead volume to the mixture for pipetting errors.

Table 18. Elution Master Mix Preparation

	# of samples	
Reagent	1	6*
EE1 (µL)	21.85	144.2
HP3 (µL)	1.15	7.59
Total Volume (µL)	23	151.79

*10% dead volume added.

6. Vortex SMB3 to resuspend the beads.
7. Add 62.5 µL SMB3 to each pool, pipette slowly to fully resuspend beads.
8. Incubate tube strip(s) in the thermocycler at 58 °C for 15 minutes.
9. Immediately after the above incubation, quick spin the tube strip(s) and place it on a magnetic stand.
10. When the liquid is clear, aspirate and discard the supernatant, remove the tube strip(s) from the magnetic stand.
11. Add 50 µL preheated EEW, vortex the tube strip(s) to resuspend the beads.
12. Place the tube strip(s) on a thermocycler, close the lid, and incubate for 5 minutes at 58 °C.
13. Remove the tube strip(s) from the heat block, centrifuge briefly.
14. Immediately place on a magnetic stand.
15. When the liquid is clear (2 minutes), aspirate and discard the supernatant, remove the tube strip(s) from the magnetic stand.
16. Repeat steps 11-15 2 times for a total of 3 washes.
17. After the third wash, add 50 µL preheated in EEW to the beads, vortex to resuspend.
18. Transfer 50 µL resuspended beads to a new set of PCR tube strip(s), vortex to resuspend.
19. Place the tube strip(s) on a thermocycler, close the lid, and incubate for 5 minutes at 58 °C.
20. Remove the tube strip(s) from the heat block, centrifuge briefly.
21. Immediately place on a magnetic stand and wait until the liquid is clear (2 minutes).
22. Using a pipette set to 50 µL, remove and discard all supernatant from each tube.
23. Quick spin the tube strip, place it back on the magnetic stand.
24. Use a P20 pipettor to remove any residual liquid from each sample.
25. Add 23 µL Elution Master Mix (step 5) to each tube, and then vortex at high speed three times for 10 seconds each.
26. Incubate the tube at room temperature for 2 minutes.
27. Quick spin the tube strip(s) and place on a magnetic stand and wait until the liquid is clear (2 minutes).
28. Transfer 21 µL supernatant to a new set of tube strip(s).
29. Add 4 µL ET2, pipette a few times to mix and centrifuge briefly.

SAFE STOPPING POINT: Store the library pools frozen at -20 °C for up to 7 days.

7.4 PCR Amplification of Enriched Libraries

1. Retrieve Enhanced PCR Mix (EPM) and PCR Primer Cocktail (PPC) included in the Respiratory Pathogen ID/AMR Enrichment Kit from the -20 °C freezer and thaw in a 2-8 °C refrigerator or on ice. Invert the reagent tube 5 times to mix; centrifuge briefly, then place on ice.
2. Retrieve AMPure XP Beads and RSB from a 2-8 °C refrigerator and thaw at room temperature for at least 30 minutes prior to using.
3. Add 5 µL PPC to each tube
4. Add 20 µL EPM to each tube and mix by pipetting. Cap tubes with a strip cap.
5. Centrifuge the tube strip(s) briefly, and place into a thermocycler and start Enriched Libraries Amplification Program PCR program (see Table 20).

Table 20. Enriched Libraries Amplification Program

Step	Temp (°C)	Time	Cycle #
1: Initial denaturation	98	30 sec	1
2: Denaturation	98	10 sec	14
3: Primer annealing	60	30 sec	
4: Extension	72	30 sec	
5: Final extension	72	5 min	1
6: Final hold	10	∞	1

Total time: ~35 minutes. Cover temperature: 100 °C.

SAFE STOPPING POINT: Store the PCR products refrigerated at 2-8 °C for up to 2 days or leave on the thermocycler for up to 24 hours.

7.5 Post-PCR Purification

1. After the Enriched Libraries Amplification programs reaches the final hold temperature, remove the tube strip(s) from thermocyclers and centrifuge briefly.
2. Ensure that AMPure XP Beads have been at room temperature for at least 30 minutes. Immediately before use, vortex briefly until the beads are thoroughly resuspended.
3. Add 90 µL of AMPure XP Beads to each tube, vortex on highest speed 3 times for 10 seconds each.
4. Let the tube strip(s) stand at room temperature for 5 minutes, then centrifuge briefly.
5. Place the tube strip(s) on a magnetic stand and let stand for 1 minute.
6. Prepare a fresh solution of 80% ethanol using 8 parts of 100% ethanol and 2 parts of MBG water.
7. When the liquid is clear, carefully remove and discard all supernatant (~140 µL) with a multi-channel P-200 pipette, ensuring not to disrupt the beads on the side of the tube.
8. Keeping the tube strip(s) on the magnetic stand, add 175 µL of 80% ethanol with a multi-channel P-200 pipette and let stand for 30 seconds. Do not mix the solution.
9. Remove and discard the ethanol.
10. Perform a second wash, by adding 175 µL of 80% ethanol and let stand for 30 seconds. Do not disrupt the beads.
11. Remove and discard the ethanol.
12. Remove the tube strip(s) from magnetic stand and centrifuge briefly.
13. Place the tube strip(s) onto magnetic stand, then use a multi-channel P-20 pipette to remove all residual ethanol from each sample tube.
14. Keep the tube strip(s) on the magnetic stand, let the tubes air dry for 2 minutes.
15. Vortex RSB briefly immediately before use.
16. Remove the tube strip(s) from the magnetic stand and add 32 µL of RSB to each sample. Thoroughly mix each sample by pipetting or vortex briefly.
17. Let tubes stand at room temperature for 2 minutes then centrifuge briefly.
18. Place the tube strip(s) on a magnetic stand for 2 minutes.
19. When the liquid is clear, transfer 30 µL of the clear supernatant from each sample on the magnetic stand to a set of new, labeled sample tube strip(s).

SAFE STOPPING POINT: Store the libraries frozen at -20 °C for up to 7 days.

8. Sequencing Library Quantification Procedure

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Sequencing Library Quantification Procedure

NOTE: PCR strip tubes must be securely capped during all centrifugation and vortexing steps. Do not reuse the caps.

IMPORTANT This step of the procedure must be performed in the Post-Amplification Lab.

8.1 Library Pool Dilution

1. Retrieve all enriched library pools from **Section 7.5**, thaw (if frozen), briefly spin and place on ice.
2. Ensure that enough Tris-Tween Mix is available for library dilution, about 0.5 mL perlibrary. If not, prepare Tris-Tween Mix according to Table 30 in a 250 mL sterile bottle (VWR Scientific, 76302-836). This solution expires in 3 months. Pour Tris-Tween Mix in a reservoir for step 4.

Table 21. Tris-Tween Mix Preparation

Reagent	Volume (µL)
Tris (1M, pH 8.0)	2,500
Tween-20	125
MBG Water	247,375
Total Volume	250,000

IMPORTANT Extra caution should be taken to prevent cross-contamination among samples in the following steps.

3. Perform a 50-fold serial dilution on the enriched library pool by diluting 3 µL of higher concentration library pool with 147 µL of Tris-Tween.
4. Repeat this dilution 3 more times to achieve a qPCR template of a 1:125,000 dilution of the original library pool.

NOTE: Only the last dilution (1:125,000 dilution) is used as qPCR template.

5. Retrieve KAPA DNA Library Quantification Kit from -20 °C freezer to thaw at room temperature. Make sure primer and ROX (if required) have been added. The Quantification Kit also contains qPCR standards. Vortex and centrifuge qPCR master mix and standards briefly.

NOTE: KAPA DNA Library Quantification Kit Master Mix bottle doesn't contain primer orROX when the kit arrives. Upon opening a new kit, thaw all reagents at room temperature. Briefly vortex and centrifuge the Primer Mix and ROX (High or Low) tubes. Add 1,000 µL of the Primer Mix and 200 µL of ROX (Check manufacturer's instructions of qPCR instrument to find out if ROX is needed) to the Master Mix bottle. Mix the contents of the reagent bottle by pipetting 5 times with P-1000 pipette. Label the bottle with the date and user initial. Do not freeze-thaw this new Master Mix more than 30 times.

6. Prepare the qPCR Working Master Mix. To calculate the number of reactions, multiply the number of library pools by 3 for triplicates, add 21 reactions for the triplicates of the 6 standards and 1 NTC, then add up to 3 reactions to account for pipetting loss, as shown in Table 22. Tris-Tween Mix can be used as no template control (NTC).

Table 22. qPCR Working Master Mix Preparation

	# of Library Pools	
Reagent	1	6*
2XKAPA SYBR fast qPCR Master Mix (µL)	12.4	520.8
MBG Water (µL)	3.6	151.2
Total Volume (µL)	16	672

*Library pools plus 9 dead volumes and a set of 6 standards and 1 NTC.

NOTE: Only the 1:125,000 dilution of each sample is used as qPCR template.

7. Retrieve a 96-well qPCR Plate. Add 16 µL qPCR Working Master Mix into all wells that will be used.

8. Inoculate the qPCR plate in triplicates with 4 µL each reaction of the standard, NTC, or the diluted qPCR template for each library.

9. Cover the qPCR plate with optical film, quick spin the plate.

10. Load the plate on a qPCR instrument and run the following qPCR thermocycler program in Table 23.

Table 23. qPCR Thermocycler Program

Step	Temp (°C)	Time	Cycle #
1: Hot start	95	5 min	1
2: Denaturation	95	30 sec	35
3: Annealing and extension	60	45 sec	
4: Melt curve analysis	95	30 sec	1
	65	30 sec	
	95	30 sec	

Total time: ~1 hour 20 minutes. Cover temperature: 105 °C

8.2 qPCR Quality Control and Data Retrieval

1. The run tab should be open on the computer that controls QuantStudio 3. If not, find the run file from its saved directory and open it.
2. Under the "Results" menu, the left side is the data graph and the right side can be shifted between plate layout view or table view.
3. From the plate layout view, select standard 6 and NTC wells. If the difference of CT (Cross Threshold) is less than 3, right click the whole set of triplicates for standard 6 to omit them. Click the blue "Analyze" button to re-analyze data.
4. Under the "Export" menu, click the "Customize" button on the right. From the dialogue window, select "Results" tab. Scroll to R^2 column to check the R^2 of the standard curve.
   a. If this value is ≥ 0.99, then continue with step 6 of analysis.
   b. If this value is < 0.99, then a maximum of one complete standard (all 3 wells) and one replicate from all remaining standards may be omitted to improve R^2. Re-analyze the data after the omission, if R^2 is still < 0.99, the entire plate must be repeated.

NOTE: Steps 3-4 ensures that the standard curve for the plate is accurate. Otherwise, the whole plate needs to be repeated. Step 5 below checks the reproducibility of each triplicate set.

5. Under the "Results" menu, from the table view, sort the crossing threshold standard deviation (CTSD) column. For any set of triplicates that CTSD is more than 0.92, one outlier from that set can be omitted to reduce CTSD. After each omission, the blue "Analyze" button needs to be clicked to re-analyze the data. If the set still has a CTSD of more than 0.92, qPCR needs to be repeated for this library.
6. Once the qPCR run passes the quality control, under the "Export" menu, ensure that the "File Name" correctly reflexes the run, "File Type" is "QuantStudio", "*.xlsx". At "Location", browse to the desired export directory.
7. Under the "Export" menu, uncheck all at the content except for "Results". Click"Export on the top right corner to export the qPCR results.

8.3 Library Pool Normalization

1. Analyze the run data and collect the average molarity for each library pool, multiply by the dilution factor 125,000 for the original library concentration.

   The goal for normalization is to dilute all the library pools to the same molarity. The optimal target molarity is 1, 2, or 4 nM. Take the lowest library pool concentration in the entire run to decide a target molarity. If a library pool concentration is below 1 nM, it can be treated as 1 nM.
2. For each library pool, calculate the volume of MBG water needed to dilute 2 µL of the pool to the target molarity using the following formula:

$$MBG\ water\ volume\ (\mu L) = \frac{[qPCR\ concentration\ (nM) * 2\mu L]}{[\text{target concentration (nM)}]} - 2\mu L$$

   Apply this formula to all library pools. If the result for a pool is less than 0, treat it as 0.
3. Thaw the library pools from Section 7.5 if necessary, vortex and centrifuge briefly.
4. Clearly label the tube strip(s) using the assigned well ID numbers to identify each library pool. Label the set as Diluted Library Pools.
5. For each library pool, add the volume of MBG water resulted from the calculation in step 3.
6. Use a P-20 multichannel pipette, pipette 2 µL of Final Library Pool (from Section 7.4) and transfer into the corresponding strip tubes from step 7.
7. Cap all the tubes, vortex and centrifuge the tube strip(s). The diluted libraries can be used in the next Chapter.

9. Procedure for Sequencing of Libraries on Illumina Sequencing System

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Procedures for Pooling and Sequencing of Libraries on Illumina Sequencing System

IMPORTANT This step of the procedure must be performed in Post-Amplification Lab.

NOTE: This user guide only describes the procedure for the Illumina MiniSeq System. A similar procedure could be used for other Illumina sequencing systems.

9.1 Reagent Preparation

1. Follow manufacturer instructions to prepare sequencing kit.
2. Ensure that enough (~100 µL) working stock of 200 mM Tris (pH 7.0) is available. If not, make a new batch according to Table 24. Make 500 µL aliquots and store at room temperature. This stock is good for up to three months.

Table 24. Preparation of 200 mM Tris pH 7.0

Reagent	Volume
MBG Water (mL)	8
1 M Tris pH 7.0 (mL)	2
Total volume (mL)	10

3. Prepare a fresh working stock of 1 mL of 0.1 N NaOH diluted from 10 N NaOH, according to Table 25. Vortex and centrifuge briefly for 2 seconds.

Table 25. Preparation of 0.1 N NaOH

Reagent	Volume (µL)
MBG Water	990
10 N NaOH	10
Total volume	1000

NOTE: 0.1 N NaOH must be made fresh.

4. Ensure that enough working stock of 1.8 pM PhiX is available. Thaw, then immediately put on ice. If the PhiX is not available, follow the protocol in Appendix D to make a new working stock. This can be done concurrently with the sample library pool.

9.2 Pooling and Denaturation of Libraries

1. Make Initial Library Pool
   a. Clearly label a new 1.5 mL DNA LoBind tube (Eppendorf, 022431021) as **Initial Pool**.
   b. Combine 2 µL of each Diluted Library Pool from Section 8.5 into the Initial Pool tube.

NOTE: If more volume of the Initial Pool is required, increase the volume of each Diluted Library Pool equally, so that the volume ratio of each pool stays equal.

   c. Vortex the pooled libraries for 5 seconds to mix, then briefly centrifuge for 2 seconds.
   d. Proceed immediately to the next step. Alternatively, the pooled libraries can be frozen at -20 °C for up to one month.

2. Denature and Dilute Library Pool
   a. Clearly label a new 1.5 mL DNA LoBind tube as **Denatured Pool**.
   b. Dilute the initial pool to 1 nM according to the following table.

Table 26: Denature of the Pooled Library

Reagent	Pooled Library Concentration		
	1 nM	2 nM	4 nM
Initial library pool (µL)	5	2.5	1.25
RSB (µL)	0	2.5	3.75
Total volume (µL)	5	5	5

   c. Add 5 µL of 0.1 N NaOH to the Denature tube, vortex the solution to mix, and then centrifuge briefly.
   d. Incubate the solution for 5 minutes at room temperature.
   e. Neutralize the pooled libraries by adding 5 µL of 200 mM Tris (pH7) to the pool. Vortex and centrifuge briefly, then place on ice. Add 985 µL ice-chilled Hybridization Buffer, vortex to mix and centrifuge briefly. Keep the denatured library pool on ice afterwards.

3. Make Final Library Pool (2.0 pM)
   a. Clearly label a new 1.5 mL DNA LoBind tube as **Final Pool**.
   b. Transfer 200 µL of the ice-chilled Denatured Pool to the Final Pool tube.
   c. Add 300 µL of ice-chilled Hybridization Buffer.
   d. Add 5 µL of ice-chilled 1.8 pM Denatured PhiX (see Table 27).

Table 27. Final Pool Preparation

Reagent	Volume
Denatured Pool (µL)	200
Ice-chilled Hybridization Buffer (µL)	300
1.8 pM Denatured PhiX DNA (µL)	5
Total volume (µL)	505

e. Invert then vortex the Final Pool for 5 seconds to mix, then briefly centrifuge for 2 seconds. Immediately place the Final Pool back on ice.
f. Keep the Final Pool on ice and immediately continue to Section 9.3 “Loading of Sequencing Libraries.”

9.3 Load and Sequence Libraries

1. Loading the MiniSeq according to the manufacturer’s instructions with the MiniSeq High Output Reagent Kit (150-cycles) (for Rapid program see Appendix E).
2. Enter run parameter settings as follows:
    a. Run ID
    b. Single Read
    b. Read 1: 147 nt
    c. Index 1: 10 nt
    d. Index 2: 10 nt
3. Ensure the run is saved to the correct location, and the reagent is purged after the completion of the run.
4. Start the sequencing run.

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Appendix A Control Recommendation

External controls				
Vendor	Part	Name/Link	Organism	Controls
Zeptometrix	NATRPC2.1-BIO	Respiratory Panel 2 (RP2.1) Controls	Viruses/Bacteria	Positive control 1 and 2
Microbiologics	8247	Respiratory Control Panel (22 Targets)	Viruses/Bacteria	Positive and Negative control
G Biosciences	786-1086	Sputo-LR		Blank Control
Hardy Diagnostics	U155	Saline (0.85%)		Blank Control
Corning	46-000-CM or equivalent	Molecular Biology Grade (MBG) water		Blank Control
Internal controls				
Vendor	**Part**	**Name/Link**	**Organism**	
European Virus Archive	Ref-SKU:011V-00884	Phocine Herpes Virus (PhHV)	Phocine Herpes Virus (PhHV; DNA)	
European Virus Archive	Ref-SKU: 011V-00885	Phocine Distemper Virus (PDV)	Phocine Distemper Virus (PDV; RNA)	
Asuragen	49650	Armored RNA® Quant Internal Process Control	Synthetic RNA	
Microbiologics	*T7-UP-LEX	T7-phage **	T7 DNA Phage	
Microbiologics	*T4-UP-LEX	T4-phage	T4 DNA Phage	
Microbiologics	*MS2-UP-LEX	MS2-phage	MS2 RNA Phage	

* Please contact IDbyDNA for further ordering instructions.

** Absolute quantification assumes the use of T7 bacteriophage as Internal Control spiked at 1.21×10^7 copies per mL of sample.

Appendix B Viscous Sample Pre-Processing Procedure

IMPORTANT This step must be performed in a Pre-Amplification Lab. The sample pipetting steps must be performed in a **biosafety cabinet**.

NOTE: When a sample requires pre-processing, this procedure is performed once for both DNA and RNA extractions.

1. Retrieve sample(s) to be pre-processed, thaw (if frozen), and place on ice.
2. Retrieve Sputo-LR (G Biosciences, 786-1086) from the 2-8 °C refrigerator and thaw at room temperature, vortex to mix.
3. Estimate the volume of the sample, add 10% (v/v) of Sputo-LR to the sample.
4. Vortex at the maximum speed for 30 seconds.
5. Incubate the mixture for 20 minutes at room temperature.
6. If the sample is still too viscous to pipette, add 4X sample volume of the sterile 0.85% saline (Hardy Diagnostics, U155), vortex to mix well.

Appendix C Concentrating Pre-Enriched Library Pool

1. Ensure that the AMPure XP beads have been at room temperature for at least 30 minutes prior to using.
2. Take sufficient volume of pre-enriched library to ensure 200 ng mass each in the desired pool.
3. Vortex the AMPure XP Beads to resuspend, add 1.8 times volume of the AMPure XP Beads to each pre-enriched library, vortex for 10 seconds.
4. Let the beads-sample mixture stand for 5 minutes.
5. Prepare fresh 80% ethanol during the incubation.
6. After the incubation, quick spin the tube strip(s), and place on the magnetic stand.
7. When the liquid in sample is clear, aspirate and discard the supernatant.
8. Add 200 µL of 80% ethanol without disrupting the beads.
9. Let all the samples stand on the magnetic stand for 30 seconds.
10. Aspirate and discard the supernatant without disturbing the beads.
11. Repeat steps 8-10 once for a total of 2 washes.
12. After the last wash, quick spin the tube and aspirate all the residual ethanol with a P20 pipettor.
13. Air-dry the tube strip(s) for 5 minutes on the magnetic stand.
14. Add 8 µL of RSB to each sample, pipette to mix, vortex for 10 seconds.
15. Incubate for 2 minutes at room temperature, quick spin.
16. Place the tube strip(s) on a magnetic stand.
17. When the liquid is clear, transfer 7.5 µL of the supernatant to a new strip tube for hybridization.

Appendix D Preparation of PhiX Working Stock

1. Thaw PhiX stock solution (10 nM) (Illumina 15017666). Vortex and centrifuge for 5 seconds.
2. Pipette 2 µL of the PhiX stock, transfer to a DNA LoBind tube (Eppendorf 22431021). Add 18 µL MBG water to dilute the stock to 1 nM.
3. Add 20 µL of freshly prepared 0.1 N NaOH (Section 9.1). Vortex and centrifuge briefly.
4. Let incubate at room temperature for 5 minutes.
5. Neutralize the pooled libraries by adding 20 µL of 200 mM Tris (pH7) (Section 9.1). Vortex the solution to mix for 5 seconds, then centrifuge briefly.Immediately place the solution on ice.
6. Dilute with 940 µL of pre-chilled HT1.
7. Transfer 117 µL to a new DNA LoBind tube and dilute with an additional 1,183 µL pre-chilled HT1, to make a final stock of 1.8 pM. Invert and vortex to mix, place back on ice.
8. Aliquot into 100 µL each and store at -20 °C. This stock is good for up to eight weeks after preparation.

Appendix E MiniSeq Rapid Program Loading

1) Use Sequencing Reagent MiniSeq Rapid Reagent Kit (100 cycles, 20044338).
2) At the software home page, click on “Manage Instrument” tab.
3) In the “Manage Instrument” page, select “System Customization”.
4) In the “System Customization” page, select the “Enable Custom Recipes” checkbox then save.
5) On the software home page, select the “Sequence” tab.
6) On the Run Setup page, select the “Manual” radio button, click “Next” to proceed.
7) On “Run Setup” interface, enter run parameter settings as follows:

a) Run ID
b) Single Read
c) Read 1: 101 nt
d) Index 1: 10 nt
e) Index 2: 10 nt

8) Browse to desired folder for the run directory; click “Next” to proceed.
9) Prepare reagent cartridge and flow cell.
10) Load consumables to the instrument.
11) Select “[Custom] MiniSeq Rapid High” from the “Recipe” Drop-down box and click “Next” to proceed.

675 Arapeen Dr, Suite 100, Salt Lake City, UT 84108 | Phone: 833-EXPLIFY (833-397-5439)
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