Nov 03, 2025

Metagenomic sequencing protocol for respiratory virus detection and sequencing from negative SARS-CoV-2 rapid antigen tests

DOI
https://dx.doi.org/10.17504/protocols.io.4r3l29pqjv1y/v1

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.4r3l29pqjv1y/v1

Protocol Citation: Emmanuela Jules, Alaa Ahmed, Hannah Dakanay, Anne Piantadosi MD, PhD 2025. Metagenomic sequencing protocol for respiratory virus detection and sequencing from negative SARS-CoV-2 rapid antigen tests. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.4r3l29pqjv1y/v1**

Manuscript citation:
Jules, E., Decker, C., Bixler, B. J., Ahmed, A., Zhou, Z. C., Arora, I., Tafesse, H., Dakanay, H., Bombin, A., Wang, E., Ingersoll, J., Bifulco, K., Frediani, J. K., Parsons, R., Sullivan, J., Greenleaf, M., Waggoner, J. J., Martin, G. S., Lam, W. A., & Piantadosi, A. (2024). Respiratory virus detection and sequencing from negative SARS-CoV-2 rapid antigen tests. *medRxiv : the preprint server for health sciences*, 2024.08.19.24311993. https://doi.org/10.1101/2024.08.19.24311993

Protocol status: Working
We use this protocol and it's working

Created: February 01, 2025

Last Modified: November 03, 2025

Protocol Integer ID: 119419

Keywords: molecular epidemiology, respiratory tract infections, public health surveillance, SARS-CoV-2, carrier RNA depletion, cDNA synthesis, Nextera, DNAse, random priming, first-strand synthesis, second-strand synthesis, metagenomic sequencing, respiratory virus detection, protocol for respiratory virus detection, nasopharyngeal swab sample, rapid antigen test, rna extraction, methods for rna, negative sar, rna, carrier rna depletion, virus, sequencing protocol

Abstract

This protocol outlines methods for RNA metagenomic sequencing to identify and characterize viruses, which we apply here to nasopharyngeal swab samples obtained from negative SARS-CoV-2 rapid antigen tests. Steps include: RNA extraction, carrier RNA depletion, cDNA synthesis (Superscript IV, ThermoFisher), and library tagmentation/indexing/amplification (Nextera XT, Illumina). Methods are based on doi: 10.3791/54117 and doi: 10.1128/mBio.01143-21

Materials

RNA Extraction reagents

	A	B	C
	Reagent	Vendor	Catalog
	EZ1 DSP Virus Kit	Qiagen	62724

Carrier RNA depletion reagents

	A	B	C
	Reagent	Vendor	Catalog Number
	Linear Acrylamide (5 mg/ml)	ThermoFisher Scientific	AM9520
	NaCl (5 M), RNase-free	ThermoFisher Scientific	AM9760G
	Tris (1M), pH 7.0, RNase-free	ThermoFisher Scientific	AM9850G
	Tris (1M), pH 8.0, RNase-free	ThermoFisher Scientific	AM9855G
	RNAClean XP, 40 mL	Beckman Coulter Life Sciences	A63987
	MgCl2 (1M)	ThermoFisher Scientific	AM9530G
	100% EtOH	Decon Labs, Inc.	V1016G
	Oligo(dT)20 Primer (50 µM) (SuperScript IV First-Strand Synthesis System)	ThermoFisher Scientific	18091200
	Hybridase Thermostable Rnase H	Biosearch Technologies	H39500

	A	B	C
	RNase-free Buffer RDD (RNase-Free DNase Set)	Qiagen	79254
	Rnase-free Dnase I (2.72 U/µl) (RNase-Free DNase Set)	Qiagen	79254
	SUPERase-In Rnase Inhibitor (20 U/µl)	ThermoFisher Scientific	AM2694
	Nuclease-free water	ThermoFisher Scientific	AM9937
	ERCC RNA spike-in dilutions	NIST Store	SKU: 2374 Note: These are in-vitro transcribed RNA from ERCC plasmids sourced from NIST.

DNase treatment reagents

	A	B	C
	Reagent	Vendor	Catalog Number
	10X reaction Buffer (Heat&Run gDNA Removal Kit)	ArcticZymes Technologies	80200-250
	HL-dsDNase (Heat&Run gDNA Removal Kit)	ArcticZymes Technologies	80200-250

cDNA Synthesis reagents

	A	B	C
	Reagent	Vendor	Catalog Number
	Nuclease-free water	ThermoFisher Scientific	AM9937

	A	B	C
	Random hexamers (50 ng/µL) (SuperScript IV First-Strand Synthesis System)	ThermoFisher Scientific	18091200
	5X RT buffer (SuperScript IV First-Strand Synthesis System)	ThermoFisher Scientific	18091200
	DTT (0.1M) (SuperScript IV First-Strand Synthesis System)	ThermoFisher Scientific	18091200
	dNTP mix (10 mM) (SuperScript IV First-Strand Synthesis System)	ThermoFisher Scientific	18091200
	SUPERase-In Rnase Inhibitor (20 U/µl)	ThermoFisher Scientific	AM2694
	SuperScript IV RT (200 U/µL) (SuperScript IV First-Strand Synthesis System)	ThermoFisher Scientific	18091200
	NEBNext Second Strand Synthesis (dNTP-free) Reaction Buffer (10 X)	New England Biolabs	B6117S
	E. coli DNA Ligase (10 U/µl)	New England Biolabs	M0205L
	E. coli DNA Polymerase I (10 U/µl)	New England Biolabs	M0209L
	E. coli Rnase H (5 U/µl)	New England Biolabs	M0297L
	AMPure XP Reagent	Beckman Coulter Life Sciences	A63881
	Tris (1M), pH 8.0, RNase-free	ThermoFisher Scientific	AM9855G
	EDTA (0.5 M), pH 8.0, RNase-free	ThermoFisher Scientific	AM9260G

Illumina Nextera XT reagents

	A	B	C
	Reagent	Vendor	Catalog Number
	Amplicon Tagment Mix (Nextera XT DNA Library Preparation Kit (96 samples))	Illumina	FC-131-1096
	Tagment DNA Buffer (Nextera XT DNA Library Preparation Kit (96 samples))	Illumina	FC-131-1096
	Neutralize Tagment Buffer (Nextera XT DNA Library Preparation Kit (96 samples))	Illumina	FC-131-1096
	Nextera PCR Master Mix (Nextera XT DNA Library Preparation Kit (96 samples))	Illumina	FC-131-1096
	IDT® for Illumina® DNA/RNA UD Indexes Sets A, B, C and D, Tagmentation (96 Indexes, 96 Samples)	Illumina	20027213, 20027214, 20027215, 20027216 (Note from Illumina: These index kits are "being discontinued. We will continue accepting orders until Mar 30, 2026 or until inventory is depleted." 20091654, 20091656, 20091658, and 20091660 are the recommended replacement kits, respectively.)

KAPA Library Quantification reagent kit

	A	B	C
	Reagent	Vendor	Catalog Number
	Complete kit (Universal)	Roche	07960140001

Troubleshooting

Before start

To minimize environmental contamination, three separate areas of the laboratory are used to: 1) prepare reagents, 2) handle samples and pre-amplified material, and 3) handle amplified material. There is a unidirectional workflow, with one individual working exclusively in the reagent area and another individual working with samples. In the protocol below, all steps are performed in the reagent area, except when noted as “sample area” or “post-amplification area".

RNA Extraction from nasopharyngeal swabs used in SARS-CoV-2 rapid antigen testing

2h

1

2h

Note

This extraction protocol uses the Qiagen EZ1 Advanced XL Instrument and Qiagen EZ1 DSP Virus Kit: https://www.qiagen.com/us/products/diagnostics-and-clinical-research/solutions-for-laboratory-developed-tests/ez1-dsp-virus-kit-na

Add Linear Acrylamide to the AVE Buffer, multiplying each volume of reagent by the number of samples and overage volume of 15%:

	A	B
	Reagents	Vol. (µL)
	Linear Acrylamide	3.6
	AVE Buffer	54
	Total (µL)	57.6

Extraction Protocol:

	A
	1. Label 2.0 mL unskirted conical-bottom sample tubes. Add 250 uL of Qiagen Buffer G2 Digestion buffer to each tube.
	2. Remove the swab from the BinaxNow cartridge. Swirl it in buffer for 10 secs. Squeeze the swab on the side of the sample tube while removing the swab from the buffer.
	3. Wipe the outside of the instrument with 70% EtOH, and insert the EZ1 Advanced XL DSP Virus Card into the card slot.
	4. Turn on instrument and run the cleaning operation. Clean the piercing unit with 70% EtOH.
	5. Press START to start work table setup. Select 200 uL sample and 60 uL elution volumes.
	6. Insert loaded cartridge rack into the EZ1 instrument: a. Invert reagent cartridges (RCV) 3 times and tap the cartridges to deposit the

	A
	reagents. Slide 1 cartridge rack per sample into the cartridge rack until it clicks into place. b. Load empty 2.0 mL unskirted conical bottom tubes into the heating system slot of each cartridge.
	7. Insert loaded tip rack into the EZ1 instrument: a. First row: 1.5 mL conical-bottom elution tubes b. Second row: Tip holders containing tips c. Third row: 1.5 mL conical bottom tube containing linear acrylamide and AVE buffer solution d. Fourth row: Samples from Step 2
	8. Close the instrument door and press START to run EZ1 Virus Protocol.
	9. Aliquot 30uL from the elution tubes into a new 1.5 mL conical bottom tube.

2

Carrier RNA Depletion (optional step if samples were extracted with carrier RNA instead of linear acrylamide)

3h 2m

3 Prepare each of the following reagent mixes separately. (These volumes are sufficient for up to 40 RNA samples including any controls. If more samples are processed, increase the volume proportionately.)

3.1 Linear Acrylamide (LA) Water - make this first

5m

	A	B
	Reagent	Vol. (µl)
	Linear Acrylamide	16
	Nuclease-Free H2O	2000

3.2 1 M Tris-HCl pH 7.5 (1000 µl) - make this second

5m

	A	B
	Reagent	Vol. (µl)
	Tris-HCl pH7	700
	Tris-HCl pH8	300

3.3 5X Hybridization Buffer (100 µl) 5m

	A	B
	Reagent	Vol. (µl)
	5M NaCl	20
	1 M Tris-HCl (pH 7.5)	50
	LA Water	30

3.4 10X RNase H Reaction Buffer (100 µl) 5m

	A	B
	Reagent	Vol. (µl)
	1 M Tris-HCl (pH 7.5)	50
	5 M NaCl	20
	1 M MgCl2	20
	LA Water	10

4 Prepare RNAClean Reagents: 5m

	A	B
	RNA SPRI Beads	135 µl per sample
	71% EtOH	7.1 mL 100% EtOH + 2.9 mL nuclease-free water
	EDTA	5 µl per sample

5 Prepare Hybridization Mix, multiplying each volume of reagent by the number of samples and overage volume of 15%: 7m

	A	B
	Reagents:	vol. (µl)

	A	B
	5X Hybridization Buffer	2
	LA Water	0.72
	Oligo dT (1500 ng stock)	1
	Total Volume	3.72

5.1 Sample Area: 10m

	A
	1. Turn on thermocycler and label a 96-well microplate to hold the RNA samples.
	2. Aliquot 5.28 µl of each RNA sample as well as 5.28 µl of Nuclease-free water as an NTC into the labeled microplate.
	3. Add 1 µl of RNA spike-in dilutions (1 pg/µl in LA water) to each sample. (See note in Materials section regarding synthesis of these RNA controls.)
	4. Add 3.72 ul of Hybridization Mix to each sample and incubate using the directions described below for Hybridization.

5.2 Cycling Conditions: 30m

	A	B
	Temp	Time
	95°C	2 min
	Ramp to 45°C	-0.1°C/sec
	45°C	hold until next step

6 Prepare the RNase Mix and distribute into a strip tube, multiplying each volume of reagent by the number of samples and overage volume of 15%: 5m

	A	B
	Reagents:	vol. (µl)
	10X Rnase H Reaction Buffer	2

	A	B
	Hybridase Thermostable Rnase H (5 U/µl)	3
	LA Water	5
	Total Volume	10

6.1 Sample Area: 10m

	A
	1. Preheat the strip tubes containing the RNase Mix at 45°C on a heat block for 2 minutes. Warm a plate holder on another heat block at 45°C to be used for keeping the sample plate warm.
	2. Remove the sample plate from the thermocycler and immediately place it in the preheated plate holder. Add 10 µl of pre-heated RNase Mix to each sample. Mix well and place back on thermocycler.
	3. Incubate sample plate using the conditions described below for RNase and immediately place on ice after sample incubation concludes. Proceed directly to DNase treatment.

6.2 Cycling Conditions:

	A	B
	Temp	Time
	45°C	30 min

7 Prepare DNAse Mix, multiplying each volume of reagent by the number of samples and overage volume of 15%: 5m

	A	B
	Qiagen RDD	7.5
	Qiagen Rnase-free Dnase I (2.72 U/µl)	2
	SUPERase-In Rnase Inhibitor (20 U/µl)	1

	A	B
	LA Water	39.5
	Total Volume	50

7.1 1. Sample Area: 40m

	A
	1. Add 50 µl of DNase Mix to each sample and incubate using the conditions described below for DNase.
	2. During DNase incubation, take SPRI beads out of the refrigerator to warm them to room temperature.
	3. After DNase incubation finishes, add 5 µl 0.5 M EDTA to each sample to stop the reaction
	4. Perform a 1.8X RNAClean XP SPRI bead clean-up

7.2 Cycling Conditions:

	A	B
	Temp	Time
	37°C	30 min

7.3 RNA SPRI Procedure: 50m

	A	B	C	D
	RNA SPRI Procedure	vol. (µl)	Wait time (minutes)	Notes
	1) Add 1.8X RNA SPRI Beads, MIX WELL	135	5	mix halfway through
	2) Place on magnet		5	
	3) Remove buffer			
	4) EtOH wash	200	1	
	5) Remove EtOH			

	A	B	C	D
	6) EtOH Wash	200	1	
	7) Remove EtOH, dry sample		10	wait until beads no longer appear glossy, but instead look matte (this may take fewere than 10 minutes)
	8) Resuspend beads in LA Water, MIX WELL	11	10	mix halfway through
	9) Place on magnet		5	
	10) Remove 10 µL from each well and transfer volume to a new strip tube or 96-well plate for cDNA synthesis. Store at -80°C until use.			

Random Priming and cDNA Synthesis

4h 40m

8 Random Priming: Aliquot random primers, multiplying each volume of reagent by the number of samples and overage volume of 15%. Vortex and spin.

5m

	A	B
	Reagents	vol. (µl)
	Random Primers	1

Random Priming and cDNA Synthesis

4h 40m

8.1 Sample Area:

15m

	A
	1. If samples have not undergone carrier RNA depletion, aliquot 5.28 µl of each sample into a strip tube or plate, and add 3.72 µl of water and 1 µl of RNA spike-in dilutions to each sample. If samples have undergone carrier RNA depletion, aliquot the total volume after depletion (10 µl) into a strip tube or plate, then add random primers to each sample and proceed with step 2.

	A
	2. Heat the strip tube or plate on thermocycler random priming program using the conditions described below for random priming. After incubation, immediately place samples on an ice block. Let the samples sit on the ice block for at least 1 min then spin down before next step.

8.2 Cycling Conditions:

	A	B
	Temp	Time
	65ºC	5 min

9 First Strand Synthesis Mix: Make First Strand Synthesis Mix, multiplying each volume of reagent by the number of samples and overage volume of 15%.Vortex and spin: 5m

	A	B
	Reagents	vol. (µl)
	5x SSIV Buffer	4
	0.1 M DTT	1
	10 mM dNTP mix	1
	dH2O	1
	SUPERase-In (20 U/µl)	1
	Superscript IV, RT (Add last)	1
	Total volume:	9

9.1 Sample Area: 1h

	A
	1. Add 9 µl of First Strand Synthesis Mix to each sample.
	2. Carefully mix samples with First Strand Synthesis Mix. Spin samples down and place them on thermocycler. Run the program using the conditions described below for First Strand Synthesis.
	3. After sample incubation is done, spin samples for 1 minute and proceed to next step.

Cycling Conditions:

	A	B
	Cycling Conditions	
	Temp.	Time
	23°C	10 min
	50°C	30 min
	80°C	10 min

10 Second Strand Synthesis Mix: Make Second Strand Synthesis Mix, multiplying each volume of reagent by the number of samples and overage volume of 15%. Vortex and spin: 10m

	A	B
	Reagents:	vol. (µl)
	Rnase-free water	43
	10X NEB Second Strand Buffer	8
	10 mM dNTP mix	3
	E. coli DNA Ligase (10 U/µl)	1
	E. coli DNA Polymerase I (10 U/µl)	4
	E. coli Rnase H (5000 U/mL)	1

	A	B
	Total volume:	60

10.1 Sample Area: 2h 15m

	A
	1. Add 60 µl of Second Strand Mix to each sample. Carefully mix and spin briefly.
	2. Place samples on thermocycler and run using the conditions described below for Second Strand Synthesis.
	3. Remove samples from thermocycler. Spin for 1 minute.
	4. Add 5 µl EDTA to stop the reaction. Mix by pipette.
	Protocol can be paused after EDTA addition and stored at -20°C overnight
	6. 30 minutes before 2nd strand incubation ends, remove SPRI beads from fridge to warm to room temperature. After the Second Strand incubation finishes, perform a 1.8X DNAClean XP SPRI bead clean-up.

Cycling Conditions:

	A	B
	Temp.	Time
	16°C	120 min

10.2 Prepare DNAClean Reagents: 50m

	A	B
	DNA SPRI Beads	153 µl per sample
	71% EtOH	7.1 mL 100% EtOH + 2.9 mL nuclease-free water
	EDTA	5 µl per sample
	Elution Buffer (EB)	1:100 ratio of Tris pH 8 to nuclease free water

Sample Area:

DNA SPRI Procedure:

	A	B	C	D
	DNA SPRI Procedure	vol. (µl)	Wait time (minutes)	Notes
	1) Add 1.8X SPRI Beads, MIX WELL	153	10	mix halfway through
	2) Place on magnet		5	
	3) Remove buffer			
	4) 71% EtOH wash	150	1	
	5) Remove EtOH			
	6) 71% EtOH Wash	150	1	
	7) Remove EtOH, dry sample		10	wait until beads no longer appear glossy, but instead look matte (this may take fewer than 10 minutes)
	8) Resuspend beads in EB, MIX WELL	11	10	mix halfway through
	9) Place on magnet		5	
	10) Aliquot 4 µl of each sample into a new strip tube or 96-well plate for Nextera. The samples can be stored at -20°C if not proceeding immediately.			

	A	B	C	D
	11) Transfer remaining 6 µl of sample into a labelled screw-cap tube and store at -80°C.			

Nextera

1h 55m

11 Prepare Tagmentation Reagent Mix (on ice block), multiplying each volume of reagent by the number of samples and overage volume of 15%: 20m

	A	B
	Reagents:	vol. (µl)
	Amplicon Tagment Mix	1
	Tagment DNA Buffer	5
	Total volume:	6

Additionally, prepare an aliquot of NT buffer at room temperature, multiplying the volume of reagent by the number of samples and overage volume of 15%:

	A	B
	Reagents:	vol. (µl)
	Aliquot Neutralize Tagment Buffer (room temp)	2.5

Then, prepare a mix of Nextera PCR Reagent Mix and water, multiplying each volume of reagent by the number of samples and overage volume of 15%:

	A	B
	Reagents:	vol. (µl)
	NPM	7.5
	Water	2.5
	Total volume:	10

Lastly, prepare DNAClean Reagents:

	A	B
	DNA SPRI Beads	42 µl per sample
	71% EtOH	7.1 mL 100% EtOH + 2.9 mL nuclease-free water
	EB	1:100 ratio of Tris pH 8 to nuclease free water

11.1 Sample Area: 45m

	A
	1. Turn on thermocycler.
	2. Take out samples and indexes from -20°C to thaw.
	3. If not previously done, aliquot 4 µl of cDNA into a 96 well plate or strip tube and place on an ice block.
	4. Add 6 uL of Tagmentation Reagent Mix to each sample. Flick to mix and spin down.
	5. Place on thermocycler following cycling conditions described below for Tagmentation.
	6. Spin thawed tubes containing unique indexes for 2 minutes.
	7. Allow NT buffer to come to room temperature.
	8. Remove samples from thermocycler and spin for 1 minute.
	9. Add 2.5 µl of Neutralize Tagment (NT) buffer to stop reaction. Mix by pipette.
	10. Incubate at room temp for 5 minutes.
	11. Vortex, and centrifuge sample for 1 minute.
	12. Place samples on ice block and add 10 µl of NPM+water mix to each reaction.
	13. To each sample, add 2.5 µl of a unique dual index
	14. Vortex and centrifuge for 1 minute.
	15. Transfer the samples on an ice block in a closed container to the post-amplification area.
	16. Perform PCR with the conditions described below for the Nextera program.
	17. After Nextera incubation finishes, add 35 µl of EB to each sample to bring the volume up to 60 µl, then perform a 0.7X DNAClean XP SPRI bead clean-up.

Tagmentation Cycling Conditions:

	A	B
	Temp	Time
	55°C	5 min
	10°C	Hold

Nextera Cycling Conditions:

	A	B	C
	Temp	Time	Cycles
	72°C	3 minutes	1
	95°C	30 sec	1
	95°C	10 sec	16 (Steps 5-7)
	55°C	30 sec	16 (Steps 5-7)
	72°C	30 sec	16 (Steps 5-7)
	72°C	5 minutes	1
	10°C	Hold	
	*** If low sample quality is expected, run for 18 cycles		

DNA SPRI Procedure:

	A	B	C	D
	DNA SPRI Procedure	vol. (µl)	Wait time (minutes)	Notes

	A	B	C	D
	1) Add 0.7X SPRI Beads, MIX WELL	42	10	mix halfway through
	2) Place on magnet		5	
	3) Remove buffer			
	4)71% EtOH wash	150	1	
	5) Remove EtOH			
	6) 71% EtOH Wash	150	1	
	7) Remove EtOH, dry sample		10	wait until beads no longer appear glossy, but instead look matte
	8) Resuspend beads in EB, MIX WELL	11	10	mix halfway through
	9) Place on magnet		5	
	10) Transfer 10 µl eluent to a screw cap tube and store at -20°C until sequencing or pooling.			

Quantification, Pooling, and Sequencing

5h

12 KAPA Quantification:

2h

Thaw the KAPA reaction mix and qPCR standards. Standard concentrations are as follows:

	A	B
	KAPA Std Curve (pM)	
	STD 1	20
	STD 2	2
	STD 3	0.2
	STD 4	0.02
	STD 5	0.002

	A	B
	STD 6	0.0002
	NTC	NTC

Prepare the KAPA qPCR mix in a 1.5 ml tube on an ice block as listed below, multiplying the volume of each reagent by the number of samples, including the 6 standards and 1 negative control, and overage volume of 15%. Vortex and spin down.

	A	B
	Reagents	vol. (µl)
	2X Kapa Mix	12
	ROX LOW	0.4
	Total volume:	12.4

Add 12.4 µl of mix to each well in an optical 96-well plate on an ice block, and then 8 µl of the NTC and 6 standards to the appropriate wells.

Sample Area: Make dilutions of the libraries with EB, and then water. Make a 1:100 dilution with EB, then make a further 1:100 dilution with water (final dilution will be 1:10,000). Add 8 µl of the final 1:10,000 dilution of each sample to the appropriate well.

Seal plate, gently vortex, and spin down. Run the plate under the following cycling conditions:

	A	B	C
	Temp.	Time	Cycles
	95°C	5 min	1
	95°C	30 sec	35
	60°C	45 sec	35
	95°C	15 sec	1
	60°C	15 sec	1
	95°C	15 sec	1

12.1 Pooling: After KAPA qPCR, note the respective concentrations of each sample in nM. Normalize each sample to the desired concentration (typically 4 nM based on Illumina calculations). Make the necessary dilutions to each sample, pooling the libraries to equimolar concentrations. If the total volume of the pool is too low, the total pool volume may be brought up to 50 µl with EB. Then perform a 0.8X SPRI.

3h

Sample Area: Perform DNA SPRI Procedure:

Warm SPRI beads to room temperature (30 min) before using.

	A	B	C	D
	DNA SPRI Procedure	vol. (µl)	Wait time (minutes)	Notes
	1) Add 0.8x SPRI Beads, MIX WELL	40	10	mix halfway through
	2) Place on magnet		5	
	3) Remove buffer			
	4) 71% EtOH wash	300	1	
	5) Remove EtOH			
	6) 71% EtOH Wash	300	1	
	7) Remove EtOH, dry sample		10	wait until beads no longer appear glossy, but instead look matte (may take fewer than 10 min)
	8) Resuspend beads in EB, MIX WELL	11	10	mix halfway through
	9) Place on magnet		5	
	10) Transfer 10 ul of eluent to a labeled cryotube for long-term storage at -20°C.			

To quantify the clean pool, perform another KAPA qPCR as outlined in **Step 11** go to step #12 , making the appropriate dilutions. Once the pool has been quantified, proceed to sequencing and prepare the pool according to manufacturer's instructions of the respective sequencing kit.