Jan 21, 2026

WWSCAN Probe-based hybridization of wastewater for metagenomic analysis of viruses

DOI
https://dx.doi.org/10.17504/protocols.io.e6nvw41w7lmk/v1

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Protocol Citation: Alessandro Zulli, Dorothea Duong, Bridgette Hughes, Vikram Chan-Herur, David Catoe, Alex Jaffe, Abigail Paulos, Amanda Bidwell, Marlene Wolfe, Alexandria Boehm 2026. WWSCAN Probe-based hybridization of wastewater for metagenomic analysis of viruses. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvw41w7lmk/v1

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

Created: August 28, 2025

Last Modified: January 21, 2026

Protocol Integer ID: 225762

Keywords: metagenomic analysis of virus, complex wastewater microbiome, wastewater sample filtration, metagenomic sequencing, detection of diverse viral taxa, hybridization of wastewater, bacterial rrna guide rna, cas9 technology with bacterial rrna guide rna, ribosomal contamination from the abundant bacterial community, wastewater sample, contamination common in wastewater matrice, using ceres nanotrap microbiome, wastewater, metagenomic approach, wastewater matrice, metagenomic analysis, diverse viral taxa, method for viral detection, environmental microbiology research application, ribosomal contamination, analysis from wastewater sample, pathogen kit, enrichment with the twist comprehensive viral research panel, viral detection, comprehensive characterization of viral community, magmax viral, abundant bacterial community, twist comprehensive viral research panel, generation sequencing, viral particle concentration, wwscan probe, sequencing method, using crispr, rrna depletion, enzymatic fragmentation, virus, targe

Abstract

This protocol describes a comprehensive metagenomic sequencing method for viral detection and analysis from wastewater samples using next-generation sequencing. The workflow begins with wastewater sample filtration through 0.45 µm filters to remove larger particles, followed by viral particle concentration using Ceres Nanotrap Microbiome A particles in a KingFisher automated system. Total nucleic acids are extracted using the MagMAX Viral/Pathogen kit, with subsequent PCR inhibitor removal via the Zymo OneStep kit to address contamination common in wastewater matrices. Library preparation involves enzymatic fragmentation, end repair, dA-tailing, and adapter ligation using the Twist Library Preparation kit. Libraries undergo targeted enrichment with the Twist Comprehensive Viral Research Panel through hybridization capture, enabling detection of diverse viral taxa (>3,000) within the complex wastewater microbiome.A critical step includes rRNA depletion using CRISPR-Cas9 technology with bacterial rRNA guide RNAs to reduce ribosomal contamination from the abundant bacterial community in wastewater. Final libraries are amplified, quantified using KAPA qPCR, pooled at equimolar ratios, and sequenced on Illumina platforms with paired-end reads ≥150 bp. This metagenomic approach enables comprehensive characterization of viral communities in wastewater, supporting public health surveillance, outbreak monitoring, and environmental microbiology research applications.

Materials

Before starting:
- Resuspend 18.6 nmol of Random Primer 6 in 372 µL nuclease free water.
- Molecular biology grade ethanol
- Molecular biology grade water
- Qiagen elution buffer
- Ceres Nanotrap Microbiome A Particles
- Ceres Nanotrap Enhancement Reagent 1 Solution
- Thermo MagMAX Microbiome Lysis Solution
- Thermo MagMAX Viral/Pathogen Nucleic Acid Isolation Kit
- Thermo Scientific Nalgene Rapid-Flow Sterile Disposable Filter Units 0.45 µm, PES, 50 mm, 150 mL
- Zymo OneStep PCR Inhibitor Removal Kit
- Random Primer 6 (S1230S)
- ProtoScript II First Strand cDNA Synthesis Kit (E6560L)
- NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module (E6111L)
- Twist Library Preparation EF Kit 2.0 (104207)
- Twist Comprehensive Viral Research Panel Kit (103550)
- Silicon-A-HRC plate
- Zymo Elution Plate
- Prep Solution (from Zymo OneStep PCR Inhibitor Removal Kit)
- Centrifuge capable of 3,500 x g
- Thermal cycler
- KingFisher Elution Plate
- Kapa HyperPure Beads
- Magnetic rack/plate (to capture beads)
- Qubit fluorometer and Qubit assay reagents (for QC)
- Twist Universal Adapters with UMIs (to be diluted to 0.3X prior to use)
- Frag/AT Buffer (for fragmentation, end repair, and dA-tailing)
- Frag/AT Enzyme (for fragmentation, end repair, and dA-tailing)
- Ligation Master Mix (for Universal Adapter Ligation)
- Nuclease Free Water
- Twist UDI Primer
- Equinox Library Amp Mix (2X)
- Universal Blocker Solution (for elution)
- Blocker Solution
- Universal Blockers
- Hybridization Mix (preheated and cooled)
- Probe Solution (for enrichment hybridization)
- Streptavidin Binding Beads
- Hybridization Enhancer
- Heat block
- 1.5 mL microcentrifuge tubes

- Magnetic stand
- Binding Buffer
- Standard Wash Buffer (68°C)
- 48°C Wash Buffer
- 0.2 mL thin-walled PCR strip-tube
- Amplification Primers, ILMN
- Jumpcode/CRISPRclean Stranded Total RNA Prep with rRNA Depletion (#KIT1014)
- RNase Inhibitor
- KAPA Illumina Library Quantification kit
- Elution buffer
- Twist CVRP Panel (Twist Comprehensive Viral Research Panel, CVRP)

Before start

Resuspend Random Primer 6

1. Resuspend 18.6 nmol of Random Primer 6 in 372 µL nuclease free water.

Sample Filtration and Viral Particle Concentration

1 Transfer 50 mL of samples (raw wastewater or liquid supernatant from wastewater solids) into a new Nalgene Rapid-Flow Sterile Disposable Filter Units 0.45 µm, PES filter.

2 Turn on the vacuum until all samples have passed through the filter.

3 Add 4.875 mL of filtered liquid sample to one well (one well per sample) of a new KingFisher 24 Well Deep Well Plate.

4 Add another 4.875 mL of filtered liquid sample to the same well location on a second KingFisher 24 Well Deep Well Plate.

5 Add 50 µL of Nanotrap Enhancement Reagent 1 (ER1) Solution to each well used.

6 Add 75 µL of Nanotrap Microbiome A Particles to each well used on the two KingFisher 24 Well Deep Well sample plates.

7 Prepare the “Lysis Plate”: Add 450 µL of MagMAX Microbiome Lysis Solution to each well of a new KingFisher 24 Well Deep Well Plate.

8 Run KF-008-WW-Nanotrap-24.bdz.

9 Once the protocol is completed, the “Lysis Plate” will contain lysate that is ready to proceed to nucleic acid extraction.

Nucleic Acid Extraction

10 Prepare the following plates for processing:

10.1 Wash Buffer Plate: Transfer 1000 µL of Wash Buffer to each well of the KingFisher Deepwell Plate.

10.2 80% Ethanol: Transfer 1000 µL of 80% ethanol to each well of the KingFisher Deepwell Plate.

10.3 Elution Plate: Transfer 60 µL of Elution Solution to each well of the KingFisher Deepwell Plate.

11 Prepare Binding Bead Mix by combining 530 µL of Binding Solution and 20 µL of Total Nucleic Acid Magnetic Beads per sample into a 50 mL Falcon Tube.

12 Invert to mix the Binding Bead Mix.

13 Transfer 400 µL of lysate to each well of a new KingFisher Deepwell Plate.

14 Add 10 µL of Proteinase K to each well.

15 Add 550 µL of Binding Bead Mix to each well.

16 Set up, load, and run the KingFisher for the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit.

17 Once the protocol is complete, the nucleic acid will be in the Elution Plate and ready to proceed to the inhibitor removal steps.

Inhibitor Removal

18 Mount a Silicon-A-HRC plate onto an Elution Plate.

19 Add 150 µL Prep Solution to the wells by piercing the cover foil in the middle.

20 Incubate at room temperature for 5 min.

21 Centrifuge the plate at 3,500 x g for 5 min.

22 Transfer 60 µL of eluate from the KingFisher Elution Plate into the wells of a prepared Silicon-A-HRC Plate mounted on a new Zymo Elution Plate.

23 Centrifuge the plate at 3,500 x g for 3 minutes.

24 The cleaned up nucleic acid is now in the Zymo Elution Plate.

cDNA Synthesis

25 Transfer 15 µL of nucleic acid into a new plate.

26 To each sample add: 5 µL Random Primer 6.

27 Incubate:

Table 1. Thermal Cycler Program for Primer Annealing

Steps	Cycles	Temp (°C)	Time
1	1	95	5 min
2	-	4	Hold

Lid Temp = 105°C, Total Volume = 20 µL

28 Make a master mix for first strand synthesis. Per sample there should be: 25 µL ProtoScript II Reaction Mix and 5 µL ProtoScript II Enzyme Mix.

29 Incubate:

Table 2. First Strand Synthesis			
Steps	**Cycles**	**Temp (°C)**	**Time**
1	1	25	5 min
2	1	42	1 hour
3	1	80	5 min
4	-	4	Hold

Lid Temp = 105°C, Total Volume = 50 µL

30 Make a master mix for second strand synthesis. Per sample there should be: 18 µL Nuclease Free Water, 8 µL NEBNext Second Strand Synthesis Reaction Buffer and 4 µL NEBNext Second Strand Synthesis Enzyme Mix.

31 Incubate:

Table 3. Second Strand Synthesis			
Steps	**Cycles**	**Temp (°C)**	**Time**
1	1	16	1 hour
2	-	4	Hold

Lid Temp = 30°C, Total Volume = 80 µL

32 Add 96 µL Kapa HyperPure Beads to each well.

33 Mix well by pipetting up and down at least 10 times.

34 Incubate at room temperature for 5 minutes.

35 Place the plate on a magnet to capture the beads.

36 Incubate until the liquid is clear.

37 Carefully remove and discard the supernatant.

38 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

39 Incubate the plate on the magnet at room temperature for 30 seconds.

40 Carefully remove and discard the ethanol.

41 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

42 Incubate the plate on the magnet at room temperature for 30 seconds.

43 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

44 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

45 Remove the plate from the magnet.

46 Resuspend the beads in 27 µL elution buffer.

47 Incubate the plate at room temperature for 2 minutes.

48 Place the plate on a magnet to capture the beads.

49 Incubate until the liquid is clear.

50 Transfer the 25 µL of clear supernatant to a new plate.

51 QC samples using Qubit.

Library Preparation

52 Normalize samples to 25 ng to 25 µL (1 ng/µL).

53 Make a master mix, for Fragmentation, End Repair, and dA-Tailing. Per sample there should be: 15 µL Nuclease Free Water, 4 µL Frag/AT Buffer and 6 µL Frag/AT Enzyme.

54 Incubate

Table 4. Frag, End Repair, dA-Tailing

Steps	Cycles	Temp (°C)	Time
1	1	4	Hold
2	1	30	7 min 30 sec
3	1	65	30 min
4	-	4	Hold

Lid Temp = 75°C, Total Volume = 50 µL

55 Dilute Twist Universal Adapters with UMIs to a final concentration of 0.3X.

56 Add 2.5 µL diluted Twist Universal Adapters with UMIs to each well.

57 Make a master mix, for Universal Adapter Ligation. Per sample there should be: 2.5 µL Nuclease Free Water and 20 µL Ligation Master Mix.

58 Incubate:

Table 5. Universal Adapter Ligation			
Steps	**Cycles**	**Temp (°C)**	**Time**
1	1	20	15 min
2	-	4	Hold

Lid Temp = 30°C, Total Volume = 75 µL

59 Add 60 µL Kapa HyperPure Beads to each well.

60 Mix well by pipetting up and down at least 10 times.

61 Incubate at room temperature for 5 minutes.

62 Place the plate on a magnet to capture the beads.

63 Incubate until the liquid is clear.

64 Carefully remove and discard the supernatant.

65 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

66 Incubate the plate on the magnet at room temperature for 30 seconds.

67 Carefully remove and discard the ethanol.

68 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

69 Incubate the plate on the magnet at room temperature for 30 seconds.

70 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

71 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

72 Remove the plate from the magnet.

73 Resuspend the beads in 17 µL elution buffer.

74 Incubate the plate at room temperature for 2 minutes.

75 Place the plate on a magnet to capture the beads.

76 Incubate until the liquid is clear.

77 Transfer the 15 µL of clear supernatant to a new plate.

78 Add 10 µL Twist UDI Primer to each well.

79 Add 25 µL Equinox Library Amp Mix (2X) to each well.

80 Incubate:

Table 6. Library PCR			
Steps	**Cycles**	**Temp (°C)**	**Time**
1	1	98	45 sec
2	12	98	15 sec
3		60	30 sec
4		72	30 sec
5	1	72	1 min
6	-	4	Hold

Lid Temp = 105°C, Total Volume = 50 µL

81 Add 40 µL Kapa HyperPure Beads (0.8X) to each well.

82 Mix well by pipetting up and down at least 10 times.

83 Incubate at room temperature for 5 minutes.

84 Place the plate on a magnet to capture the beads.

85 Incubate until the liquid is clear.

86 Carefully remove and discard the supernatant.

87 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

88 Incubate the plate on the magnet at room temperature for 30 seconds.

89 Carefully remove and discard the ethanol.

90 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

91 Incubate the plate on the magnet at room temperature for 30 seconds.

92 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

93 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

94 Remove the plate from the magnet.

95 Resuspend the beads in 32 µL elution buffer.

96 Incubate the plate at room temperature for 2 minutes.

97 Place the plate on a magnet to capture the beads.

98 Incubate until the liquid is clear.

99 Transfer the 30 µL of clear supernatant to a new plate.

Jump Code rRNA Depletion

100 Obtain the Bacterial rRNA Guide RNA and thaw the contents on ice.

101 Transfer 4.3 µL of Bacterial rRNA Guide RNA per reaction into a strip tube.

102 Incubate:

Table 7. Guide RNA Pre-Heat			
Steps	**Cycles**	**Temp (°C)**	**Time**
1	1	65	2 min
2	-	0	Hold

Lid Temp = 75°C, Total Volume = 5 µL

103 Place the strip tube on ice once the incubation is completed.

104 Make a master mix for rRNA depletion. Per sample there should be: 3.9 µL pre-heated Guide RNA, 2 µL 10X Cas9 Buffer, 2.3 µL Cas9, and 1 µL RNase Inhibitor.

105 Incubate at room temperature for 10 minutes.

106 Once the incubation is complete, add 9.2 µL of the master mix for rRNA depletion to 10.8 µL of pooled libraries.

107 Incubate:

Table 8. CRISPR Digestion			
Steps	**Cycles**	**Temp (°C)**	**Time**
1	1	42	60 min
2	-	0	Hold

Lid Temp = 52°C, Total Volume = 20 µL

108 Place the strip tube on ice once the incubation is completed.

109 Add 30 µL nuclease free water to the reaction.

110 Add 50 µL Kapa HyperPure Beads (1X) to each well.

111 Mix well by pipetting up and down at least 10 times.

112 Incubate at room temperature for 5 minutes.

113 Place the plate on a magnet to capture the beads.

114 Incubate until the liquid is clear.

115 Carefully remove and discard the supernatant.

116 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

117 Incubate the plate on the magnet at room temperature for 30 seconds.

118 Carefully remove and discard the ethanol.

119 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

120 Incubate the plate on the magnet at room temperature for 30 seconds.

121 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

122 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

123 Remove the plate from the magnet.

124 Resuspend the beads in 40 µL nuclease free water.

125 Incubate the plate at room temperature for 2 minutes.

126 Place the plate on a magnet to capture the beads.

127 Incubate until the liquid is clear.

128 Transfer the 40 µL of clear supernatant to a new plate.

129 Make a master mix for Jumpcode PCR. Per sample there should be: 50 µL 2X PCR Master Mix, 5 µL P5 Primer, and 5 µL P7 Primer.

130 Incubate:

Table 9. Jumpcode PCR

Steps	Cycles	Temp (°C)	Time
1	1	95	2 min
2	10	98	20 sec
3		55	30 sec
4		72	30 sec
5	1	72	2 min
6	-	4	Hold

Lid Temp = 105°C, Total Volume = 100 µL

131 Add 100 µL Kapa HyperPure Beads (1X) to each well.

132 Mix well by pipetting up and down at least 10 times.

133 Incubate at room temperature for 5 minutes.

134 Place the plate on a magnet to capture the beads.

135 Incubate until the liquid is clear.

136 Carefully remove and discard the supernatant.

137 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

138 Incubate the plate on the magnet at room temperature for 30 seconds.

139 Carefully remove and discard the ethanol.

140 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

141 Incubate the plate on the magnet at room temperature for 30 seconds.

142 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

143 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

144 Remove the plate from the magnet.

145 Resuspend the beads in 32 µL elution buffer.

146 Incubate the plate at room temperature for 2 minutes.

147 Place the plate on a magnet to capture the beads.

148 Incubate until the liquid is clear.

149 Transfer the 30 µL of clear supernatant to a new plate.

Enrichment

150 Pool up to 16 samples at 1500 ng total per pool.

151 Heat the Hybridization Mix at 65°C in the heat block for 10 minutes, or until all precipitate is dissolved, then cool to room temperature on the benchtop for 5 minutes.

152 Make a master mix of Universal Blocker Solution for elution. Per sample there should be: 5 µL Blocker Solution and 7 µL Universal Blockers.

153 Concentrate samples by performing a 1.8X SPRI cleanup.

154 Add the appropriate amount of Kapa Beads to each well.

155 Mix well by pipetting up and down at least 10 times.

156 Incubate at room temperature for 5 minutes.

157 Place the plate on a magnet to capture the beads.

158 Incubate until the liquid is clear.

159 Carefully remove and discard the supernatant.

160 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

161 Incubate the plate on the magnet at room temperature for 30 seconds.

162 Carefully remove and discard the ethanol.

163 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

164 Incubate the plate on the magnet at room temperature for 30 seconds.

165 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

166 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

167 Remove the plate from the magnet.

168 Resuspend the beads in 12 µL Universal Blocker Solution.

169 Make a master mix of Probe Solution. Per sample there should be: 20 µL Hybridization Mix (preheated and cooled), 4 µL Twist CVRP Panel and 4 µL nuclease free water.

170 Heat the probe solution to 95°C for 2 minutes in a thermal cycler with the lid at 105°C, then immediately cool on ice for 5 minutes.

171 While probe solution is cooling on ice, heat the tube containing the resuspended indexed library pool at 95°C for 5 minutes in a thermal cycler with the lid at 105°C, then equilibrate both the probe solution and resuspended indexed library pool to room temperature on the benchtop for 5 minutes.

172 Vortex and spin down the probe solution, then transfer the entire volume to the resuspended indexed library pool. Mix well by vortexing.

173 Pulse-spin the tube to ensure all solution is at the bottom of the tube.

174 Add 30 µL Hybridization Enhancer to the top of the entire capture reaction.

175 Pulse-spin the tube to ensure there are no bubbles present.

176 Incubate the hybridization reaction at 70°C for 16 hours in a thermal cycler with the lid at 85°C. (70 µL total volume).

177 Vortex the pre-equilibrated Streptavidin Binding Beads until mixed.

178 Add 100 µL Streptavidin Binding Beads to a 1.5 mL microcentrifuge tube. Prepare one tube for each hybridization reaction.

179 Add 200 µL Binding Buffer to the tube and mix by pipetting.

180 Place the tube on a magnetic stand for 1 minute, then remove and discard the clear supernatant.

181 Make sure to not disturb the bead pellet. Remove the tube from the magnetic stand. Repeat the wash.

182 Repeat two more times for a total of three washes.

183 After removing the clear supernatant from the third wash, add a final 200 µL Binding Buffer and resuspend the beads by vortexing until homogenized.

184 Heat the resuspended beads at 68°C for at least 10 min.

185 After the hybridization is complete, open the thermal cycler lid and directly transfer the volume of each hybridization reaction into a corresponding tube of preheated Streptavidin Binding Beads. Mix by pipetting and flicking.

186 Incubate the tube of the hybridization reaction with the Streptavidin Binding Beads for 5 minutes at 68°C, agitation is not required.

187 Remove the tube containing the hybridization reaction with Streptavidin Binding Beads from the mixer and pulse-spin to ensure all solution is at the bottom of the tube.

188 Place the tube on a magnetic stand for 1 minute.

189 Remove and discard the clear supernatant including the Hybridization Enhancer. Do not disturb the bead pellet.

190 Remove the tube from the magnetic stand and add 200 µL 68°C Standard Wash Buffer.

191 Mix by pipetting.

192 Incubate the tube for 5 minutes at 68°C.

193 Pulse-spin to ensure all solution is at the bottom of the tube.

194 Transfer the entire volume (~200 µL) into a new 1.5 mL microcentrifuge tube, one per hybridization reaction. Place the tube on a magnetic stand for 1 minute.

195 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

196 Remove the tube from the magnetic stand and add 200 µL of 48°C Wash Buffer 2. Mix by pipetting, then pulse-spin to ensure all solution is at the bottom of the tube.

197 Incubate the tube for 5 minutes at 48°C.

198 Place the tube on a magnetic stand for 1 minute.

199 Remove and discard the clear supernatant. Make sure to not disturb the bead pellet.

200 Repeat the wash two more times, for a total of three washes.

201 After the final wash, use a 10 µL pipette to remove all traces of supernatant. Do not allow the beads to dry.

202 Remove the tube from the magnetic stand and add 45 µL water. Mix by pipetting until homogenized, then incubate this solution, hereafter referred to as the Streptavidin Binding Bead slurry, on ice.

203 If the Streptavidin Binding Bead slurry has settled, mix by pipetting.

204 Transfer 22.5 µL of the Streptavidin Binding Bead slurry to a 0.2 mL thin-walled PCR strip-tube. Keep on ice.

205 Make a master mix of Enrichment PCR. Per sample there should be: 25 µL Equinox Lib Amp Mix (2X) and 2.5 µL Amplification Primers, ILMN.

206 Incubate:

Table 10. Enrichment PCR			
Steps	**Cycles**	**Temp (°C)**	**Time**
1	1	98	45 sec
2	12	98	15 sec
3		60	30 sec
4		72	30 sec
5	1	72	1 min
6	-	4	Hold

Lid Temp = 105°C, Total Volume = 50 µL

207 Add 90 µL Kapa HyperPure Beads (1.8X) to each well.

208 Mix well by pipetting up and down at least 10 times.

209 Incubate at room temperature for 5 minutes.

210 Place the plate on a magnet to capture the beads.

211 Incubate until the liquid is clear.

212 Carefully remove and discard the supernatant.

213 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

214 Incubate the plate on the magnet at room temperature for 30 seconds.

215 Carefully remove and discard the ethanol.

216 Keeping the plate on the magnet, add 200 µL of 80% ethanol.

217 Incubate the plate on the magnet at room temperature for 30 seconds.

218 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

219 Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated.

220 Remove the plate from the magnet.

221 Resuspend the beads in 32 µL elution buffer.

222 Incubate the plate at room temperature for 2 minutes.

223 Place the plate on a magnet to capture the beads.

224 Incubate until the liquid is clear.

225 Transfer the 30 µL of clear supernatant to a new plate.

Library Pooling

226 Measure the library concentration by PCR using the KAPA Illumina Library Quantification kit.

227 Pool samples, aiming for equimolar amounts of each. Negative control libraries may not yield enough material to be pooled in the same quantity as actual samples.

Sequencing

228 Sequence the pool on an Illumina system using a kit that will yield ≥150PE reads.