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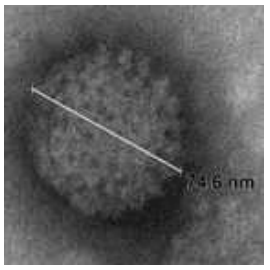
Optimized conditions for whole genome sequencing of avian reoviruses.



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We use this protocol and it's working

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Abstract

Whole genome sequencing (WGS) is becoming an essential tool to characterize the genomes of avian reovirus (ARV), an emerging pathogen and growing threat to poultry producers and integrators. This protocol details a workflow for ARV genome purification and amplification to increase the yield of ARV reads after next generation sequencing.

Guidelines

This protocol is per 6 x 10^6cells (2xT75 flasks at 95 % confluency) infected with approximately 20 µL of ARV supernatant and incubated (38°C, humidified, with 5% CO_2) for 5 days, but it is scalable to larger or smaller cell lysates. Adjust volumes according to relative number of cells

Materials

LIST OF REAGENTS

Virion purification

NaCl (Millipore Sigma, catalog number S9888)
1M $MgCl_2$ (Millipore Sigma, catalog number 63069)
100 mM Tris pH 7.4 (Millipore Sigma, catalog number 648315)
Molecualr grade H_2O
Capto Core 700 resin (GE Healthcare, catalog number GE17-5481-01)
Illustra MicroSpin column (GE Healthcare, catalog number GE27-3565-01)

RNA extraction

MagMAX™ Viral RNA Isolation Kit (Applied Biosystems, catalog number AM1939)
Isopropanol

Host rRNA depletion

Custom ssDNA probes (IDT)
NEBNext Probe Hybridization Buffer (New England BioLabs, catalog number E6314)
RNase H enzyme (New England Biolabs, catalogue number M0297S)
DNase I enzyme (New England Biolabs, catalog number M0303S)
RNAClean XP beads (Beckman Coulter, catalog number A63987)

Reovirus Single Primer Amplification (R-SPA)

SuperScript IV Reverse Transcriptase kit (ThermoFisher Scientific, catalog number 18090010)
Klenow polymerase kit (NEB, product number M0210S)
10mM dNTP mix (New England BioLabs, catalog number N0447S)
Agencourt AMPure XP beads (Beckman Coulter, product number A63880)
HiFi PCR Phusion kit (New England Biolabs, catalog number M0530S)
70% ethanol (nuclease-free)
Nuclease-free H_2O
Primer R-8N: 5’-AGCTTTTAGAATCCTCAGAGGNNNNNNNN-3’ (IDT)
Primer R: 5’-AGCTTTTAGAATCCTCAGAGG-3’ (IDT)

Safety warnings

Personnel should wear gloves at all times.

Introduction

1 **NOTES:**

This protocol is per 6 x 10^6cells (2xT75 flasks at 95 % confluency) infected with approximately 20 µL of ARV supernatant and incubated (38°C, humidified, with 5% CO_2) for 5 days, but it is scalable to larger or smaller cell lysates. Adjust volumes according to relative number of cells

ARV collection

2 **Prepare Virus Dilution Buffer (VDB)**

2.1 List of reagents:

	A	B	C
	REAGENT	**Per 1 L**	**[Final]**
	5 M NaCl	30 ml	150 mM
	1 M MgCl2	15 ml	15 mM
	100 mM Tris pH 7.4	1 ml	10 mM
	ddH2O	To 1L	

Filter-sterilize through standard 0.45µm bottle filter and store at room temperature

3 **Preparing cell lysates**

3.1 Harvest infected LMH cells in their own supernatant with a cell scraper (Corning™ catalogue number
3011) and centrifuged at 3000 x g for 10 mins at room temperature (RT).

3.2 Resuspended the pellet in 350 µL of VDB.

3.3 Sonicate the infected cells on ice: 3 pulses at 30% amplitude, 10 sec. on, 30 sec. off, we used a Branson Digital Sonifier 450 (Branson Ultrasonics Corporation).

3.4 Store the infected cell lysates for not more than a week at 4ºC.

ARV virion purification

4 This part of the protocol was adapted from the original protocol detailed in James, K.T., et al., *Novel High-throughput Approach for Purification of Infectious Virions.*Sci Rep, 2016. 6: p. 36826.

5 **Preparing 50% Capto700 Slurry**

5.1 Spin CaptoCore700 resin at 800 x g for 5 minutes.

5.2 Remove as much supernatant as possible without disturbing the resin.

5.3 Record the volume of resin left in the tube.

5.4 Add equal-to-resin volume of VDB and mix by vortexing.

5.5 Repeat steps 5.1 to 5.4.

5.6 Store as "50% slurry in VDB" at room temperature.

6 **Virion purification**

6.1 Centrifuge the infected cell lysates at 800 x g for 10 minutes to remove nuclei and debris.

6.2 Collect the supernatant in a fresh tube containing 100 µl of 50% CaptoCore 700 Slurry.

6.3 Mix sample end-over-end for 45 minutes at room temperature.

6.4 Subject sample to centrifugation at 800 x g for 10 minutes.

6.5 Transfer the top phase to a new tube containing 100 µl of 50% CaptoCore 700 Slurry.

6.6 Repeat steps 6.2 to 6.5 for increasing purity.

6.7 To clear all CaptoCore 700 resin, pass the supernatant of the last cleaning cycle through an Illustra MicroSpin column at 800 x g for 5 minutes.

6.8 Store virus at 4ºC, or aliquot and store at -20ºC for long-term usage. Avoid freeze-thaw cycles.

RNA extraction

7 We are going to use MagMAX™ Viral RNA Isolation Kit (Applied Biosystems, catalog number AM1939) to extract the RNA from the purified ARV virions.

NOTE: Spray work surfaces and pipettes with an RNase decontamination solution and wipe clean.

8 **Prepare Lysis/Binding Solution**

	A	B
	Reagent	**1 reaction**
	Lysis/Binding Soln. Concentrate	400 µl
	Carrier RNA	2 µl
	Mix briefly by pipetting and then add:	
	100% Isopropanol	400 µl
	Mix well by vortexing.	

9 **Prepare the Wash solution 1**
Add 12 mL 100% isopropanol to the bottle labeled Wash Solution 1 Concentrate and mix well. Mark the label to indicate that the isopropanol was added. Store at room temperature.

10 **Prepare the Wash solution 2**
Add 44 mL 100% ethanol to the bottle labeled as Wash Solution 2 Concentrate and mix well. Mark the label to indicate that the ethanol was added. Store at room temperature.

11 **Prepare Bead Mix**

Vortex the RNA Binding Beads at moderate speed to form a uniform suspension before pipetting.

	A	B
	Component Per	**1 reaction**
	RNA Binding Beads	10 µL
	Lysis/Binding Enhancer	10 µL

Include ~10% overage to cover pipetting error when preparing the Bead Mix.
Mix thoroughly by gently vortexing.

12 **RNA extraction**

12.1 Add 400 µL sample to **802 µL Lysis/Binding Solution** in a 2ml microcentrifuge tube.
IMPORTANT: *When adding sample, immerse pipette tips slightly in the Lysis/Binding Solution to prevent creating aerosols that can lead to cross-contamination.*

12.2 Mix by gently flicking the tube several times.

12.3 Centrifuge the mix briefly (~2 sec) to collect the contents at the bottom of the tube.

12.4 Vortex the Bead Mix at moderate speed to create a uniform suspension before pipetting

12.5 Add **20 µL Bead Mix** to each sample

12.6 Gently shake the sample for 4 min on a vortex mixer to fully lyse viruses and bind RNA to the RNA Binding Beads.

12.7 Centrifuge the samples briefly (~2 sec) to collect tube contents.

12.8 Move the samples to a magnetic stand to capture the RNA Binding Beads.

12.9 Leave the tubes on the magnetic stand for 3 minutes or until a clear bead pellet is formed.

12.10 Carefully aspirate and discard the supernatant without disturbing the beads.

12.11 Remove the Processing Tube from the magnetic stand.

12.12 Add **300 µL Wash Solution 1** to each sample and vortex at moderate speed for 30 sec.

12.13 Centrifuge briefly (~2 sec) to collect tube contents.

12.14 Leave the tubes on the magnetic stand for 5 minutes or until a clear bead pellet is formed.

12.15 Carefully aspirate and discard the supernatant without disturbing the beads.

12.16 Remove the Processing Tube from the magnetic stand.

12.17 Repeat steps 12.12 -12.15 to wash a second time with **300 µL Wash Solution 1**.

12.18 Add **450 µL Wash Solution 2** to each sample and vortex at moderate speed for 30 sec.

12.19 Centrifuge briefly (~2 sec) to collect tube contents.

12.20 Leave the tubes on the magnetic stand for 5 minutes or until a clear bead pellet is formed.

12.21 Carefully aspirate and discard the supernatant without disturbing the beads

12.22 Remove the Processing Tube from the magnetic stand.

12.23 Repeat steps 12.18 -12.21 to wash a second time with 450 µL Wash Solution 2.

12.24 Leave the tube open on the magnetic stand at room temp for 2 min to allow any remaining alcohol from the second wash with Wash Solution 2 to evaporate.

12.25 Inspect the tubes and if there is residual solution, remove as much as possible with a very fine-tipped pipettor.

12.26 Leave the tube open on the bench for another minute.

12.27 Add 30 µL Elution Buffer (room temp) to each sample, and shake or vortex vigorously for 4 min.

12.28 Leave the tubes on the magnetic stand for 5 minutes or until a clear bead pellet is formed.

12.29 Transfer 26uL of the supernatant, which contains the RNA, to a nuclease-free container appropriate for your application, and store the purified RNA at –20°C.

Host rRNA depletion

13 **Preparing custom probes**

This part of the protocol was adapted from the original protocol detailed in Parris, D.J., H. Kariithi, and D.L. Suarez, Non-target RNA depletion strategy to improve sensitivity of next-generation sequencing for the detection of RNA viruses in poultry. J Vet Diagn Invest, 2022. **34**(4):p. 638-645.
The sequence of the probes can be found in the manuscript.

13.1 Dilute the probes to 100 µM stocks.

13.2 Pool equal volumes (1 µl) of each probe to make the working master mix for the depletion protocol.

13.3 Aliquot the master mix depending on the number of samples that are going to be frequently sequenced

14 **ssDNA probes hybridization**

We are going to hybridize a set of custom probes to the chicken rRNA and mitochondrial RNA.

14.1 Prepare RNA-Probe master mix per sample

	A	B
	Custom ssDNA probe pool (10 µM)	1 µl
	Probe Hybridization Buffer	2 µl

14.2 Add 3 µl of the above mix to 12 µl of extracted RNA (final vol. 15 µl)

14.3 Mix by pipetting up and down 10 times

14.4 Spin down briefly in a tabletop centrifuge to collect the volume to the bottom of the tube.

14.5 Place samples in a thermocycler with a heated lid set to 105°C, and run the following program:

	A	B
	95°C	2 min
	95°C-22°C	0.1°C/sec
	22°C	5 min

14.6 Place samples on ice and proceed immediately to the next step.

15 **RNase H digestion**

We are going to use RNase H enzyme (New England Biolabs, catalogue number M0297S) to degrade chicken and bacteria rRNA that hybridized with the ssDNA probes.

15.1 On ice, prepare the following master mix and use immediately

	A	B
	RNase H Enzyme	2 µl
	RNase H Reaction Buffer	2 µl
	Nuclease-free Water	1 µl

15.2 Add 5 µl of the master mix to the RNA sample (15 µl) from step 14.6 (final vol. 20 µl).

15.3 Mix by pipetting up and down 10 times.

15.4 Spin down briefly in a tabletop centrifuge to collect the volume to the bottom of the tube.

15.5 Place samples in a thermocycler with heated lid off) and incubate at **37°C for 30 min**.

15.6 Place samples on ice and proceed immediately to the next step.

16 **DNase I Digestion**

We are going to use DNase I enzyme (New England Biolabs, catalog number M0303S) to degrade the custom ssDNA probes from the samples.

16.1 On ice, prepare a DNase I Digestion Master Mix

	A	B
	DNase I Reaction Buffer	5 µl
	DNase I Enzyme	2.5 µl
	Nuclease-free Water	22.5 µl

16.2 Mix by pipetting up and down 10 times.

16.3 Add 30 µl of the above mix to the RNA sample from Step 15.6 (final vol. 50 µl).

16.4 Mix by pipetting up and down 10 times.

16.5 Spin down briefly in a tabletop centrifuge to collect the volume to the bottom of the tube.

16.6 Place samples in a thermocycler with heated lid off) and incubate at **37°C for 30 min**.

16.7 Place samples on ice and proceed immediately to the next step.

17 **Bead-purification of RNAs**
We are going to use RNAClean XP beads (Beckman Coulter, catalog number A63987) to clean the RNA before cDNA synthesis.

Before you start: Bring RNAClean XP beads to room temperature (RT) at least 30 mins before starting purification. **Vortex RNA sample purification beads to resuspend.** ***Prepare 80% ethanol (nuclease free). Mix 7 parts 100% ethanol with 3 parts nuclease-free H_2O. You will need 600ul 80% ethanol per sample.***

17.1 Add 110 µl (2.2X) ***RNAClean XP*** beads to 50 µl to host depleted RNA.

17.2 Mix by pipetting up and down 10 times.

17.3 Incubate samples at **RT for 15 min**.

17.4 Place the plate on magnetic stand to separate the beads from the supernatant.

17.5 After 5 min (or when the solution is clear), carefully remove and discard the supernatant.

17.6 Add 200 µl of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at **RT for 30 seconds**, and then carefully remove and discard the supernatant.

17.7 Repeat Step 17.6. Place back on the magnet and remove traces of ethanol with a p10 pipette tip.

17.8 Air dry the beads for **up to 5 min** while the plate is on the magnetic stand with the lid open.

17.9 Remove the plate from the magnetic stand and elute the RNA from the beads by adding 15 µl of nuclease-free H_2O.

17.10 Mix well by pipetting up and down 10 times. Incubate for 5 min.

17.11 Fast-spin the sample to collect the liquid from the sides of the tube.

17.12 Place the plate on the magnetic stand for 5 min.

17.13 Transfer 12 µl of the clean host-depleted RNA to a new PCR tube.

17.14 Place the tube on ice and proceed with cDNA production. Alternatively, the sample can be stored at −80°C.

cDNA production and single primer amplification

18 This part of the protocol was adapted from the original protocol detailed in Chrzastek, K., H. Sellers, and D. Kapczynski, *A universal, single primer amplification protocol (R-SPA) to perform whole genome sequencing of segmented dsRNA reoviruses*. 2021, bioRxiv.

19 **Reverse Transcription**
We are going to use SuperScript IV Reverse Transcriptase (ThermoFisher Scientific, catalog number 18090010) to perform the reverse transcription step.

19.1 Spray work surfaces with Eliminase (or similar cleaner) and wipe clean.

19.2 Combine the following on a reaction tube:

	A	B
	Primer R8N (100 µM)	1 µl
	10 mM dNTP mix	1 µl
	Nuclease free H20	1 µl

19.3 Add 10 µl of the extracted RNA to the reaction tube for a final volume of 13 µl.

19.4 Mix briefly by carefully pipetting up and down 10 times

19.5 Heat up the RNA-primer mix at **95ºC for 4 mins and incubate one ice for 1 min**

19.6 Prepare the SuperScript IV reaction (7 µl vol.):

	A	B
	5x SS IV buffer	4 µl
	100uM DTT	1 µl
	Recomb.RNase inhibitor (40U/ µl)	1 µl
	SS IV Reverse Transcriptase (200U/ µl)	1 µl

19.7 Mix briefly by carefully pipetting up and down 10 times

19.8 Add the SuperScript IV reaction (7 µl) to the RNA-primer mix (13 µl) and mix well by pipetting up and down 20 times.

19.9 Incubate the 20 µl reaction in the thermocycler under the following conditions:

	A	B
	23 ºC	10 min
	55 ºC	10 min
	80 ºC	10 min
	4 ºC	∞

NOTE: Use your first-strand cDNA immediately **to generate double-stranded cDNA** ***or store at -20 ºC.***

20 **cDNA second strand synthesis**

We are going to use Klenow polymerase (NEB,product number M0210S) to generate double-stranded (ds) cDNA.

20.1 Make a stock of primer R8N to 10 µM

20.2 Add the following to the 20µl reverse transcriptase reaction:

	A	B
	Primer R8N (10 µM)	1 µl
	10 mM dNTP mix	1 µl
	Klenow Rxn buffer	2 µl

20.3 Mix well by carefully pipetting up and down 20 times

20.4 Heat up the mix at **94ºC for 3 mins and cool down on at 4 ºC in the thermocycler**

20.5 Add 1 ul of Klenow fragment and mix well by carefully pipetting up and down 20 times

20.6 Incubate the mix at **37 °C for 60 min** (final volume, 25 µl)

21 **Double-stranded cDNA purification**

We are going to use Agencourt AMPure XP beads (Beckman Coulter, product number A63880) to purify ds-cDNA

Before you start: Bring AMPure XP beads to room temperature (RT) at least 30 mins before starting purification. **Vortex RNA sample purification beads to resuspend.** ***Prepare 70% ethanol (nuclease free). Mix 7 parts 100% ethanol with 3 parts nuclease-free H_2O. You will need 600ul 70% ethanol per sample.***

21.1 Vortex the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.

21.2 Add 45 µl AMPure XP beads (X 1.8) to the 25 µl ds-cDNA reaction

21.3 Mix well by carefully pipetting up and down 20 times. The color of the mixture should appear homogenous after mixing.

21.4 Let the mixed samples incubate for **5 minutes at RT**.

21.5 Place the reaction plate onto the magnet for 2 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.

21.6 Carefully remove and discard the supernatant (cleared solution).

21.7 With your tubes still on the magnet stand, add 200 µl of 70% ethanol to each well and incubate for **30 seconds at RT**. Then, carefully remove and discard the ethanol.

21.8 Repeat step 21.7 for a total of two washes.

21.9 Remove traces of ethanol with a 20 µl pipette and air dry the beads for up to **5 mins at RT**.

21.10 Remove the reaction plate from the magnet plate, and then add 20 µL of nuclease-free H2O to each well of the reaction plate and pipette mix 10 times. Incubate for **2 minutes at RT**.

21.11 Place the reaction plate back onto the magnet and wait for 1 minute (or until the solution is clear) to separate beads from the solution.

21.12 Transfer 15 µL the supernatant containing your clean ds-cDNA (clear solution) into a new tube.

21.13 ***NOTE: Use your ds-cDNA immediately*** **to generate double-stranded cDNA** ***or store at -20 ºC.***

22 ds-cDNA amplification by PCR

We are going to use a HiFi PCR Phusion kit (NEB, catalog number M0530S) for ds-cDNA PCR amplification.

22.1 Prepare the PCR reaction as follows (final vol. 45 µl):

	A	B
	Nuclease free H20	31 µl
	1× Phusion HF buffer	10 µl
	10 mM dNTP mix	1 µl
	primer R (10 µM)	2.5 µl

	A	B
	Phusion DNA polymerase	0.5 µl

22.2 Mix well by carefully pipetting up and down 10 times

22.3 Add 5 µl of clean ds-cDNA to each PCR reaction mix to have a final volume of 50 µl.

22.4 Place the 50 µl reaction in a thermocycler and run the PCR as follows:

	A	B	C
	Denaturation	98 °C	30 sec
	35 cycles of	98 °C	30 sec
		50 °C	30 sec
		72 °C	1 min
	Extension	72 °C	10 min
		4 °C	∞

23 **PCR product purification**

We are going to use Agencourt AMPure XP beads (Beckman Coulter, product number A63880) to purify ds-cDNA

Before you start: Bring AMPure XP beads to RT at least 30 mins before starting purification. Prepare 70% ethanol (nuclease free). Mix 7 parts 100% ethanol with 3 parts nuclease-free H_2O. You will need 600ul 70% ethanol per sample.

23.1 Vortex the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.

23.2 Add 90 µl AMPure XP beads (X 1.8) to the 50 µl PCR product

23.3 Mix well by carefully pipetting up and down 20 times. The color of the mixture should appear homogenous after mixing.

23.4 Let the mixed samples incubate for **5 minutes at RT**.

23.5 Place the reaction plate onto the magnet for 2 minutes to separate beads from the solution. Wait for the solution to clear before proceeding to the next step.

23.6 Carefully remove and discard the supernatant (cleared solution).

23.7 With your tubes still on the magnet stand, add 200 µl of 70% ethanol to each well and incubate for **30 seconds at RT**. Then, carefully remove and discard the ethanol.

23.8 Repeat step 23.7 for a total of two washes.

23.9 Remove traces of ethanol with a 20 µl pipette and air dry the beads for up to **5 mins at RT**.

23.10 Remove the reaction plate from the magnet plate, and then add 35 µL of nuclease-free H_2O to each well of the reaction plate and pipette mix 10 times. Incubate for **2 minutes at RT**.

23.11 Place the reaction plate back onto the magnet and wait for 1 minute (or until the solution is clear) to separate beads from the solution.

23.12 Transfer 30 µL the supernatant containing your clean ds-cDNA (clear solution) into a new tube.

23.13 **Now your ARV cDNAs are ready for genomic library preparation. Alternatively, they can be stored at*-20 ºC***

Protocol references

Virion purification: Modified from James, K.T., et al., *Novel High-throughput Approach for Purification of Infectious Virions.*Sci Rep, 2016. 6: p. 36826.(https://www.nature.com/articles/srep36826)

Host rRNA depletion: Parris, D.J., H. Kariithi, and D.L. Suarez, Non-target RNA depletion strategy to improve sensitivity of next-generation sequencing for the detection of RNA viruses in poultry. J Vet Diagn Invest, 2022. **34**(4):p. 638-645.

R-SPA protocol: Chrzastek, K., H. Sellers, and D. Kapczynski, *A universal, single primer amplification protocol (R-SPA) to perform whole genome sequencing of segmented dsRNA reoviruses*. 2021, bioRxiv. (https://www.biorxiv.org/content/10.1101/2021.11.01.466778v1.full)