JVDI: Supplemental material

Parris DJ, et al. Non-target RNA depletion strategy to improve sensitivity of next-generation sequencing for the detection of RNA viruses in poultry

Supplemental Table 2. Step-by-step protocol that was used for depleting non-target rRNA sequence with custom probe sets used in our study.

***Custom Primer Preparation:**

Primers were synthesized by IDT and based on the reverse complement sequence of the rRNA region of interest (tiled in non-overlapping ~120mers). IDT primers were diluted to 100 µM stocks once received. Equal volumes of each primer were pooled to make the depletion primer "mix."

Component	Volume, µL
Depletion primer mix (10 µM)	1
Probe hybridization buffer (NEB buffer #E6314 Special Order)	2
Total volume	3

1. Hybridize the probes to the RNA

1.1. Prepare an RNA/Probe master mix as follows:

1.2. Add 3 µL of the above mix to 12 µL of total RNA sample (2–10 ng/µL).

1.3. Mix by pipetting up and down ≥10 times.

1.4. Spin down briefly in a tabletop centrifuge, and immediately proceed to the next step.

1.5. Place samples in a thermocycler with a heated lid set to ~105°C, and run the following program, which will take 15–20 min to complete:

Temperature	Time
95°C	2 min
95–22°C	0.1°C/s
22°C	5 min hold

1.6. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step.

2. RNase H digestion

2.1. On ice, prepare a master mix according to the following table, and mix by pipetting up and down ≥10 times; use immediately.

Component	Volume, µL
RNase H (NEB #M0297L)	2
RNase H reaction buffer	2
Nuclease-free water	1
Total volume	5

2.2. Add 5 µL of the above mix to the RNA sample from step 1.6.

2.3. Mix by pipetting up and down 10 times.

2.4. Spin down briefly in a table-top centrifuge, and immediately proceed to the next step.

2.5. Place samples in a thermocycler (with lid at 40°C or off) and incubate at 37°C for 30 min.

2.6. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step to prevent nonspecific degradation of RNA.

3. DNase I digestion

3.1. On ice, prepare a DNase I digestion master mix according to the following table, and mix by pipetting up and down 10 times; use immediately.

Component	Volume, µL
DNase I reaction buffer	5
DNase I (RNase-free, NEB M0303L)	2.5
Nuclease-free water	22.5
Total volume	30

3.2. Add 30 µL of the above mix to the RNA sample from step 2.6.

3.3. Mix by pipetting up and down 10 times.

3.4. Spin down briefly in a table-top centrifuge, and immediately proceed to the next step.

3.5. Place samples in a thermocycler (with lid at 40°C or off) and incubate at 37°C for 30 min.

3.6. Spin down the samples in a tabletop centrifuge, and place on ice. Proceed immediately to the next step.

4. RNA purification after rRNA depletion using RNA sample purification beads (Beckman Coulter A63987)

4.1. Vortex RNA sample purification beads to resuspend.

4.2. Add 110 µL (2.2×) of resuspended RNA sample purification beads to the RNA sample. Mix well by pipetting up and down 10 times. Be careful to expel all of the liquid out of the tip during the last mix. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

4.3. Incubate samples on ice for 15 min.

4.4. Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

4.5. After 5 min (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA (Caution: do not discard the beads).

4.6. Add 200 µL of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 s, and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the RNA.

4.7. Repeat step 4.6 for a total of 2 washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet, and remove traces of ethanol with a p10 pipette tip.

4.8. Air-dry the beads for up to 5 min while the tube/plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of RNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

4.9. Remove the tube/plate from the magnetic stand. Elute the RNA from the beads by adding 8 µL of nuclease-free water. Mix well by pipetting up and down 10 times. Incubate for 2 min. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

4.10. Place the tube/plate on the magnetic stand. After 5 min (or when the solution is clear), transfer 6 µL to a new PCR tube.

4.11. Place the tube on ice and proceed with NGS library construction or other downstream application. Alternatively, the sample can be stored at –80°C.