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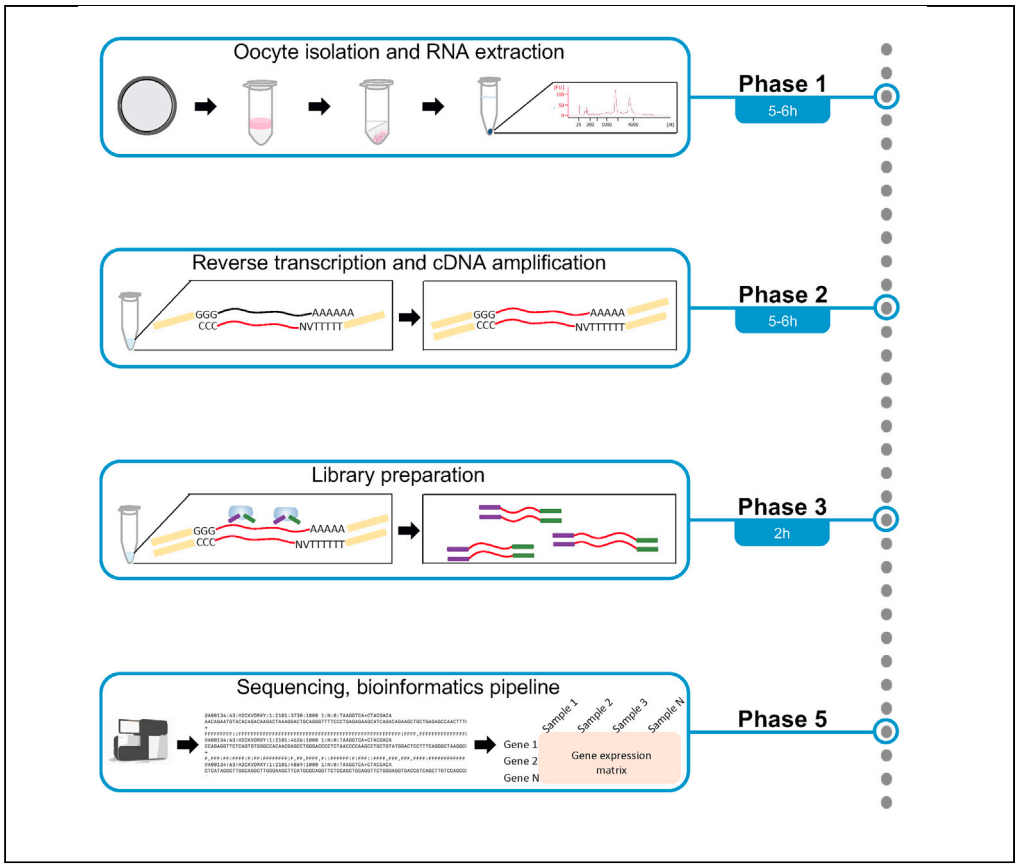
Isolation of high-quality total RNA and RNA sequencing of single bovine oocytes



Studying individual mammalian oocytes has been extremely valuable for the understanding of the molecular composition of oocytes including RNA storage. Here, a detailed protocol for isolation of oocytes, extraction of total RNA from single oocytes followed by full-length cDNA amplification, and library preparation is presented. The procedure permits the production of cost-effective and high-quality sequencing libraries. This protocol can be adapted for transcriptome analysis of oocytes from other species and be used to generate high-quality data from single embryos.

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Highlights

Isolation of high-quality total RNA from single bovine oocytes

Detailed procedures for amplification of complementary DNA for library preparation

A bioinformatic pipeline for the quantitation of transcript abundance

The protocol also enables high-quality data production from single embryos

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Isolation of high-quality total RNA and RNA sequencing of single bovine oocytes

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SUMMARY

Studying individual mammalian oocytes has been extremely valuable for the understanding of the molecular composition of oocytes including RNA storage. Here, a detailed protocol for isolation of oocytes, extraction of total RNA from single oocytes followed by full-length cDNA amplification, and library preparation is presented. The procedure permits the production of cost-effective and high-quality sequencing libraries. This protocol can be adapted for transcriptome analysis of oocytes from other species and be used to generate high-quality data from single embryos.

For complete details on the use and execution of this protocol, please refer to Biase and Kimble (2018).

BEFORE YOU BEGIN

The protocol below describes the specific steps for working with bovine oocytes, all the way from obtaining and preserving oocyte samples to the quantification of transcript abundances (see Figure 1A for overview of the procedures). However, we have also used this protocol for production of transcriptome data from single embryos. This protocol can be applied to other species such as human and mouse.

Note: Confirm that all reagents are available before beginning the protocol.

Note: Wipe all the surfaces with an RNase decontamination solution (i.e. RNAseZap or an alternative), which includes but is not limited to: bench, centrifuge, pipettors, pipette tip boxes.

⚠ CRITICAL: Use sterile and nuclease-free tubes and nuclease-free pipette tips containing a filter barrier. Always change pipette tips to avoid cross contamination, or contamination of reagents.

⚠ CRITICAL: Water and all solutions and tubes should be free of nucleases.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ampure XP	Beckman Coulter	A63881
Bovine serum albumin	Millipore Sigma	126609-5GM
Chloroform	Fisher Scientific	AAJ67241AP

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
dNTPs	Thermo Fisher Scientific	10297018
Ethanol	VWR	BP2818500
Gibco TrypLE Express Enzyme	Fisher Scientific	12604-013
Glycoblue	Thermo Fisher Scientific	AM9516
Hyaluronidase	Millipore Sigma	H4272
Isopropanol	VWR	32727-0010
Maxima H Minus RT	Thermo Fisher Scientific	EP0751
Mineral Oil	Sigma	M5310-500mL
PBS, 10X Solution	Thermo Fisher Scientific	193871
PEG 8000	VWR	101443-878
Phasemaker Tubes	Thermo Fisher	A33248
Phosphate buffer saline	Thermo Fisher Scientific	193871
RNAlater Stabilization Solution	Thermo Fisher Scientific	AM7021
RNAseZap	Thermo Fisher Scientific	AM9780
RNAse inhibitor	Promega	N2611
Terra PCR Direct Polymerase Mix	Takara	639271
TripLe Express	Gibco	12604-013
Tris EDTA buffer	VWR	BP24731
Trizol reagent	Thermo Fisher Scientific	15596026
Critical commercial assays		
Agilent High Sensitivity DNA Kit	Agilent	5067-4626
Agilent RNA 6000 Pico Kit	Agilent	5067-1513
Nextera DNA Flex Library Preparation Kit	Illumina, Inc	20015826
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33230
Oligonucleotides		
5′-AAGCAGTGGTATC AACGCAGAGT-3′	IDT	n/a
5′-AAGCAGTGGTATCAA CGCAGAGTACATrGrGrG-3′	IDT	n/a
5′-AAGCAGTGGTATCAACGCA GAGTACT30VN-3′	IDT	n/a
Software and algorithms		
Hisat2	(Kim et al., 2019)	http://daehwankimlab.github.io/hisat2/
Samtools	(Li et al., 2009; Morgan and Pagés, 2012)	http://www.htslib.org/
Biobambam	(Tischler and Leonard, 2014)	https://github.com/gt1/biobambam
Picard	n/a	https://broadinstitute.github.io/picard/
Featurecounts	(Liao et al., 2014)	http://subread.sourceforge.net/
R software	(Ihaka and Gentleman, 1996)	https://www.r-project.org/
Other		
Bioanalyzer	Agilent	G2939B
Thermocycler	Eppendorf	6331000025
Centrifuge	Eppendorf	022623508
Rotor	Eppendorf	FA-45-30-11
Qubit	Thermo Fisher Scientific	Q33238
Stripper pipette	CooperSurgical	MXL3-STR
Stripper tips	CooperSurgical	MXL3-175
BRAND® PCR Mini-cooler with transparent lid	Sigma	BR781260-2EA
DynaMag – 96 side magnet	Thermo Fisher Scientific	12331D

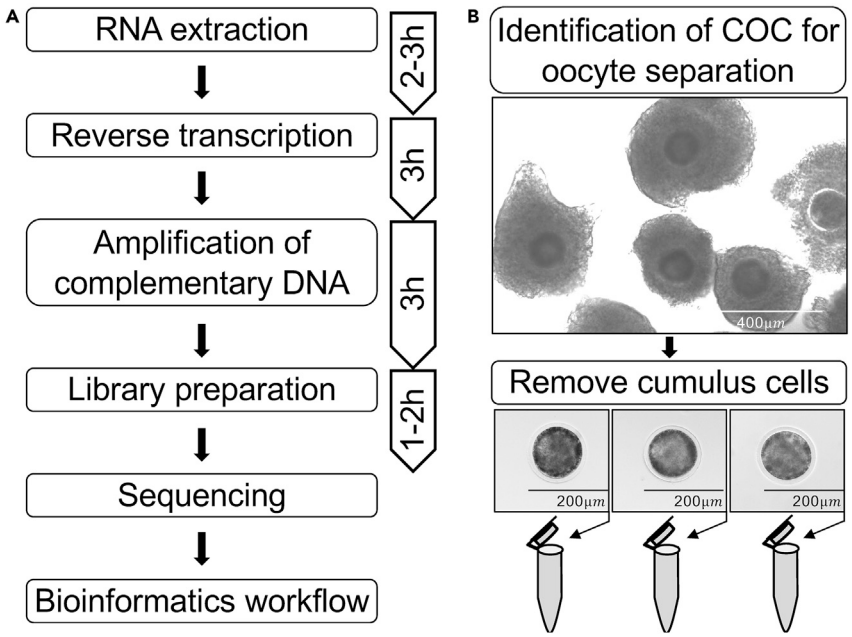

Figure 1. Single-cell RNA sequencing of oocytes

(A) Schematic diagram of the workflow following the deposit of the oocyte in the microcentrifuge tube.

(B) Representative images of the samples processed. Following the removal of the cumulus cells, single oocytes are deposited in 0.2 mL tubes with minimal volume (~ 1 μL) of PBS containing RNAse inhibitor (0.2 IU/μL) and bovine serum albumin (0.2%). Images were obtained with a 10× objective. Scale bar for COCs is 400 μm, and for oocytes is 200 μm.

MATERIALS AND EQUIPMENT

RNA elution mix

Reagent	Final concentration	Amount
Oligo-dT 5′-AAGCAGTGGTATCAACG CAGAGTACT30VN-3′ (100 μM)	10 μM	1 μL
RNase inhibitor (40 U/μL)	0.6 U/μL	0.15 μL
Water	n/a	3.75 μL
Total	**n/a**	**5 mL**

Prepare sufficient mix for two extra reactions, one will serve as a non-template control. Keep it on ice until used and do not store for future use in this protocol.

Reverse transcription mix

Reagent	Final concentration	Amount
PEG 8000 (50%)	7.5 ×	1.5 μL
Maxima RT Buffer (5×)	1 ×	2 μL
dNTPs (10mM)	1 mM	1 μL
Template switching oligonucleotide 5′-AAGCAGTGGTATCAACGCAG AGTACATrGrGrG-3′ (100 μM)	2 mM	0.2 μL
Maxima H Minus RT (200 U/μL)	5 U/μL	0.25 μL
RNAse inhibitor (40 U/μL)	0.5 U/μL	0.13 μL
Total	**n/a**	**5 μL**

Prepare sufficient mix for two extra reactions, one will serve as a non-template control. Keep it on ice until used and do not store for future use in this protocol.

PCR reaction mix		
Reagent	Final concentration	Amount
Terra direct Buffer	2×	10 μL
IsPCR (oligonucleotide) 5′-AAGCAGTGGTATCAACGCAGAGT-3′	10 μM	0.2 μL
Terra polymerase	1.25 U/μL	1 μL
Total	**n/a**	**11.2 μL**

Prepare sufficient mix for two extra reactions, one will serve as a non-template control. Keep it on ice until used and do not store for future use in this protocol.

Other solutions	
Name	Reagents
Denuding solution	1× Trypsin, RNase inhibitor (0.2U/μL)
Oocyte wash solution	1× PBS, RNase inhibitor (0.2U/μL), BSA (0.2%)
Isopropanol 50%	50% Isopropanol (v/v)
Ethanol 75%	75% Ethanol (v/v)
Ethanol 80%	80% Ethanol (v/v)

Ethanol 80% must be done on the same day of its use. It is also recommended that RNase inhibitor is added to these solutions on the day of their use. Do not store for future use in this protocol.

Alternatives: This protocol uses a Bioanalyzer to assess amplification and library quality, which can be substituted by a TapeStation.

Other brands of thermocycler can also be used if it has heated lid and holds 0.2mL centrifuge tubes.

Alternative brands of centrifuge can also be used if they are refrigerated.

STEP-BY-STEP METHOD DETAILS

Oocyte collection

Timing: [2–3 h]

Cattle (Bos taurus) oocytes can be collected from multiple sources depending on the hypothesis or the biological questions addressed in a specific study. Potential sources of oocytes are in vivo aspiration of follicles using ultrasonography-guided ovum pick up (Bo et al., 2019) or ex vivo aspiration of follicles from ovaries obtained from an abattoir (Tribulo et al., 2019). All oocytes used in this protocol were obtained from ex vivo aspiration of follicles from ovaries obtained from an abattoir. Ovaries were obtained post-mortem, and no animal was handled or euthanized for this study. Thus, this work was carried out in compliance with the Institutional Animal Care and Use Committee of Virginia Tech. We note that the user should follow the appropriate regulations when obtaining biological samples from vertebrates. Cumulus oocytes complexes (COCs) can be obtained from multiple sources.

Note: This protocol starts with the COCs (Figure 1B) after their isolation and selection for further work. In a cumulus-oocyte complex, the oocyte will be identified as a large cell, often >100 μm in diameter, enclosed in a thick layer of glycoproteins, the zona pellucida (Gupta, 2018; Hyttel et al., 1986; Wassarman and Litscher, 2018). The cumulus cells are the small cells surrounding the zona pellucida (Figure 1B).

Note: The procedures described for the handling of COCs and oocytes are carried out with the aid of a stereoscope.

1. Stripping of cumulus cells from oocytes.

a. Add three drops (50 μL) of Denuding solution on a 35mm dish and cover with oil.
b. Add three drops (50 μL) of Oocyte wash solution on a 35mm dish and cover with oil.

2. Transfer the oocytes into one of the drops with Denuding solution and pipette (20 or 100 μL pipettor) to remove the cumulus cells.
3. Using a stripper pipette (175 μm tip) aspirate the oocytes to the next drop of Denuding solution. Repeat the removal of the cumulus cells with the (20 or 100 μL pipettor) or the stripper pipette.
   a. Repeat the washing once again using the third drop of denuding solution. Aspirate as minimal volume as possible.
4. Using a stripper pipette (175 μm tip) aspirate the oocytes to the drop containing the Oocyte washing solution. Aspirate as minimal volume as possible.
   a. Transfer the oocytes to the next drop containing the Oocyte washing solution and repeat the procedure until no cumulus cells are visible under the stereoscope.
5. Using a stripper pipette (175 μm tip) aspirate one oocyte with minimal volume of washing solution and transfer into a 0.2 mL microcentrifuge tube.
6. Immerse the tube immediately into liquid nitrogen (snap freezing). Preserve the material at -80°C until used for RNA extraction.
   a. Alternatively, if liquid nitrogen is not available, transfer the oocytes to tubes containing 5 μL of an RNA stabilization solution (Camacho-Sanchez et al., 2013). Preserve the material at -80°C until used for RNA extraction.

Pause point: Cells can be maintained at -80°C for long-term storage

Total RNA extraction from single oocytes

Timing: [2–3 h]

This step involves the extraction of total RNA from single oocytes using Trizol reagent. The procedures described here have been adapted from the manufacturer's protocol to minimize the loss of total RNA obtained from single oocytes and embryos. A schematic of the procedure with pictures of critical steps is depicted in Figure 2.

Note: Pre-spin the Phasemaker Tubes for 30 sec at 12,000 × *g*.

Note: In our laboratory we work with up to 12 tubes containing single oocytes in a single batch.

⚠ CRITICAL: It is particularly important that the tubes containing the oocyte lysates do not thaw before the addition of the Trizol reagent to the tube.

7. Transfer the 0.2 mL microcentrifuge tubes containing the single oocytes to a precooled PCR rack.
8. Before the solution containing the cell lysate thaws, add 150 μL of a monophasic solution of Trizol (Chomczynski and Sacchi, 1987, 2006; Rio et al., 2010) to the 0.2 mL microcentrifuge tube. Add Trizol reagent to all tubes you are working with, then proceed to the next step.
9. Remove the tube from the cold rack onto a rack at ~24°C.
10. Assuming that a minimum volume (~ 1 μL) was added to the tube with the oocyte, add 10 μL of water to the solution, and mix the solution gently by pipetting up and down (~5–10 times). Add water to all tubes you are working with, then proceed to the next step.
11. Transfer the homogenate solution to into a 2 mL centrifuge tube containing a gel polymer capable of separating the aqueous phase from the organic phase (Murphy and Hellwig, 1996).
12. Let the solution stand at ~24°C for 5 min.
13. Add 30 μL of chloroform to the solution and mix the solution vigorously by shaking the tube for 15 s.
14. Let the solution stand for 3 min at ~24°C.

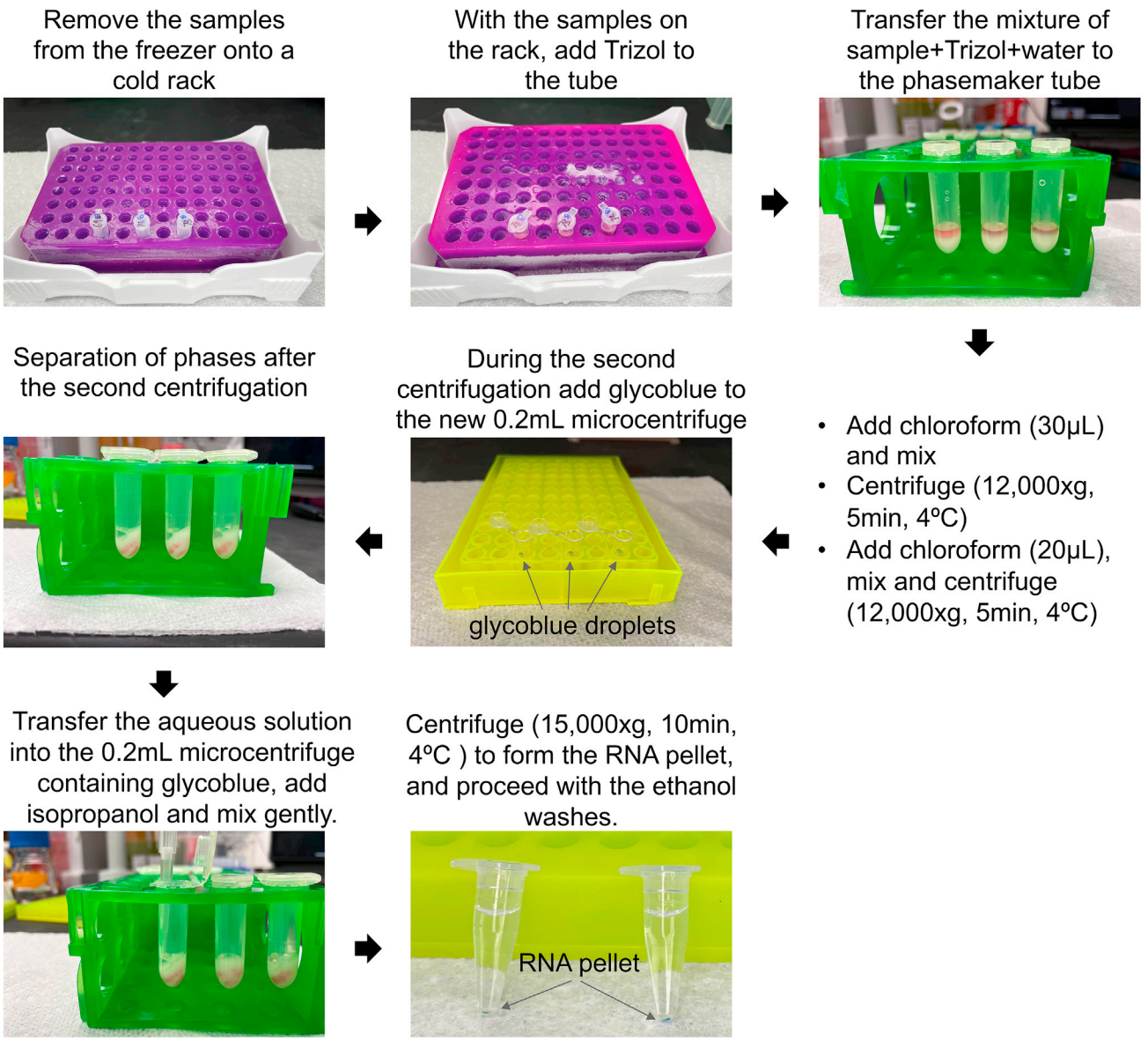

Figure 2. Schematic of the total RNA extraction from single oocytes

15. Centrifuge at 12,000 × *g* for 5 min at 4°C.
16. Add 20 µL of chloroform to the solution and mix the solution vigorously by shaking the tube for 15 s,
17. Centrifuge at 12,000 × *g* for 5 min at 4°C.
    a. In the meantime add 1µL (15 µg) of glycoblue to a new 0.2 mL microcentrifuge tube.
18. Remove all aqueous solution from the phase maker tube and place it in the 0.2 mL microcentrifuge tube containing the glycoblue.
    a. Mix the aqueous solution with the glycoblue by pipetting very gently (5 times).

Note: Do not to touch the gel with the pipette tip. If the gel is touched, dispense the aqueous solution onto the gel gently and use a new pipette tip.

Note: This step can also be executed by transferring the aqueous solution to all tubes, followed by mixing of the solution with glycoblue, which can be done with a multi-channel pipette.

19. Add 100 μL of isopropanol to the solution and mix by gentle pipetting (5 times).

Note: This step can also be executed by adding isopropanol to all tubes, followed by mixing of the solution with a multi-channel pipette.

20. Let the tube stand at ~24°C for 10 min.

Note: Extended precipitation may be used to increase the yield of total RNA. In this case store samples for 12–18h at -20°C and resume the protocol to pellet the RNA.

21. Centrifuge at 15,000 × *g* for 10 min at 4°C.

Note: If the centrifuge rotor accommodates only 2 mL tubes. Add the 0.2 mL tubes into a 0.5 mL tube, which can be placed in an adaptor or add the 0.5 mL tube into a 2mL tube. Cut the lids of the 0.5 and 2 mL tubes to facilitate the handling.

22. Remove the supernatant gently and discard. Do not disrupt the pellet.

Note: It is easier to remove most of the liquid with a 200 μL pipette and remove the remainder with a 10 μL or 20 μL pipette, which allows a more precise removal of the liquid without touching the pellet.

Note: If RNALater or equivalent high salt RNA stabilization solution was used there will a formation of a bubble-like blue precipitate (Camacho-Sanchez *et al.*, 2013). Remove the isopropanol without disrupting this bubble. Add 150 μL of isopropanol 50% to the microcentrifuge tube, and repeat steps 21 and 22.

23. Add 150 μL of Ethanol 75% to the 0.2 mL tube.
24. Centrifuge at 15,000 × *g* for 2 min at 4°C.
25. Remove the supernatant gently and discard. Do not disrupt the pellet.
26. Add 150 μL of Ethanol 75% to the 0.2 mL tube.

Pause point: The pelleted RNA can be stored long term in Ethanol 75% at -80°C without cause RNA degradation.

⚠ CRITICAL: Only continue the protocol if the reverse transcription and PCR amplification will be executed without interruption or storage of nucleic acids synthesized.

Note: If the material was stored resume the protocol on step 27.

27. Centrifuge at 15,000 × *g* for 2 min at 4°C.
28. Remove the supernatant gently and discard. Do not disrupt the pellet.

Note: It is easier to remove most of the liquid with a 200 μL pipette and remove the remainder with a 10 μL or 20 μL pipette, which allows a more precise removal of the liquid. Remove ethanol 75% as much as possible without touching the pellet.

⚠ CRITICAL: Have the RNA elution mix ready before starting air drying the pellet.

29. Air-dry the pellet, which takes about one minute if all the liquid is removed. Proceed immediately to the desired assay using the RNA.

⚠ CRITICAL: Proceed immediately to the reverse transcription using the RNA.

⚠ CRITICAL: Avoid extensive air drying because it will make the elution of the pellet very difficult. The pipetting required for the dissolution of a dry pellet will increase the breaking of the RNA strands.

Note: Since the RNA used for sequencing is limited in quantity, assessment of quality prior to reverse transcription is not possible. Alternatively, assess the RNA quality of some samples that are collected and processed exclusively for quality control. Add 1 μL of water directly to the RNA pellet and wait for the pellet to dissolve in approximately 5–10 min. on ice. Proceed with the automated capillary electrophoresis.

Reverse transcription

Timing: [2 h]

This protocol for synthesis of complementary DNA was adapted from the molecular crowding single-cell RNA barcoding sequencing (mcSCRB-seq) (Bagnoli et al., 2018) and the Smart-Seq2 (Picelli et al., 2013, 2014). The procedure described here rely on manual liquid handling and transfer, however the protocol can be adapted for robotic liquid handlers (Jaeger et al., 2020).

⚠ CRITICAL: Spin down all microtubes after thawing reagents (in the case of enzymes, spin down the tubes in a refrigerated centrifuge immediately before pipetting the necessary volume), before and after incubations.

⚠ CRITICAL: Use sterile and nuclease-free pipette tips containing a filter barrier. Always change pipette tips to avoid cross contamination.

Note: Have all the programs set up on the thermocycler prior to starting the protocol.

30. Add 5 μL the RNA elution mix directly onto the pellet and allow the total RNA pellet to dissolve in this solution. Maintain the tubes on ice.

Note: This step usually takes approximately 5 min. Do not speed up the elution by pipetting. Only proceed once the pellet disappears.

31. Using a thermocycler, incubate the solution at 72°C for 3 min.
32. Immediately after the conclusion of the previous step, immerse the tubes on ice. Keep the tubes on ice for 3–5 min.
33. While the tubes are on ice, prepare the Reverse transcription mix, keep tubes on ice.
34. Add 5 μL of Reverse transcription mix to the solution containing RNA, mix gently by pipetting (~5 times).
35. Using a thermocycler, incubate the solution at 42°C for 90 min.

Note: Due to the presence of PEG 8000, the Reverse transcription mix is viscous. Start preparation of the reverse transcription mix by adding the buffer to help the dispensing of the PEG 8000. Mix this solution very gently. On step 34 mix the solution very gently.

Cleanup of the DNA:RNA hybrids

Timing: [1 h]

Note: Remove the magnetic beads from the refrigerator 30 minutes prior to using it. At the time of use, vortex the magnetic beads to mix the solution well. In our laboratory we prepare aliquots of the beads solution to prevent contamination of the bottle.

Note: All procedures for DNA:RNA clean-up are carried out at ~24°C. Magnetic beads are magnetic nano spheres coated with a biopolymer exhibiting high affinity to nucleic acids (see review by (Berensmeier, 2006))

36. Add 10 μL of magnetic beads solution to each tube. After the addition of magnetic beads to all tubes, mix the solution with gentle pipetting (~ 10 times). Let the tubes stand for 5 min.
37. Transfer the tubes with magnetic beads to a magnetic rack. Let the tubes stand for 8 min.
38. Remove the solution gently without disrupting the beads and discard the solution. Use a 100 μL pipette set at > 20 μL to remove the ~ 20 μL of volume.
39. With the tubes still on the magnetic rack, gently add 200 μL of ethanol 80% to avoid disrupting the magnetic beads.
    a. Wait for 30 s
40. Remove the solution carefully to avoid disrupting the magnetic beads
41. Gently add 200 μL of ethanol 80% to avoid disrupting the magnetic beads.
    a. Wait for 30 s
42. Remove the solution carefully to avoid disrupting the magnetic beads
43. Remove the tubes from the rack and let the tubes standing on a regular rack or the beads to dry.

Note: Usually, it takes ~5 min for "cracks" to form on the beads, which indicates their dryness.

44. Add 9.5 μL of water to each tube, making sure that the water is deposited on the magnetic beads that are on the wall of the microcentrifuge tube.
    a. Wait for ~2 min for the magnetic beads to begin absorbing water.
45. Mix the solution gently with a 10 μL pipette (~ 5 times).
    a. Let the tubes stand for 2 min.
46. Transfer the tubes to the magnetic rack.
    a. Let the tubes stand for 5 min.
47. Very gently, aspirate 9 μL of the solution containing the DNA:RNA hybrids into a new 0.2 mL tube.

Note: Avoid aspirating beads. If beads are seen in the pipette tip, dispense the liquid gently, wait for 2 min and repeat the process.

Full-length transcript polymerase chain reaction

Timing: [2 h]

48. Prepare a PCR reaction mix. Add 11 μL to each tube and mix by gentle pipetting (~5 times).
49. Carry out the amplification using the following cycles.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	15 s	8–11 cycles
Annealing	66°C	15 s	
Extension	68°C	4 min	
Final extension	72°C	4 min	1
Hold	4°C	Forever	

Note: Ideally, the number of cycles should be determined by assaying the RNA from few oocytes at different cycle numbers to avoid over-amplification, which can be observed by the presence of peaks when assaying the amplified complementary DNA on the automated capillary electrophoresis.

50. Remove the tubes from the thermocycler and proceed with the clean-up.

Amplified DNA cleanup

Timing: [1 h]

Note: Remove the magnetic beads from the refrigerator 30 minutes prior to using it. At the time of use, vortex the magnetic beads to mix the solution well.

Note: All procedures for DNA clean-up are carried out at ~24°C

51. Add 20 μL of magnetic beads solution to each tube. After the addition of magnetic beads to all tubes, mix the solution with gentle pipetting (~ 10 times). Let the tubes stand for 5 min.
52. Transfer the tubes with magnetic beads to a magnetic rack. Let the tubes stand for 8 min.
53. Remove the solution gently without disrupting the beads and discard the solution. Use a 100 μL pipette set at > 40 μL to remove the ~40 μL of volume.
54. With the tubes still on the magnetic rack, gently add 200 μL of ethanol 80% to avoid disrupting the magnetic beads.
    a. Wait for 30 s
55. Remove the solution carefully to avoid disrupting the magnetic beads
56. Gently add 200 μL of ethanol 80% to avoid disrupting the magnetic beads.
    a. Wait for 30 s
57. Remove the solution carefully to avoid disrupting the magnetic beads
58. Remove the tubes from the rack and let the tubes stand on a regular rack for the beads to dry.

Note: Usually, it takes ~5 min for "cracks" to form on the beads, which indicates their dryness.

59. Add 10.5 μL of Tris EDTA buffer to each tube, making sure that the buffer is deposited on the magnetic beads that are on the wall of the microcentrifuge tube.
    a. Wait for ~2 min for the magnetic beads to begin absorbing water.
60. Mix the solution gently with a 10 μL pipette (~ 5 times).
    a. Let the tubes stand for 2 min.
61. Transfer the tubes to the magnetic rack.
    a. Let the tubes stand for 5 min.
62. Very gently, aspirate 10 μL of the solution containing the DNA:RNA hybrids into a new 0.2 mL tube.

Note: Avoid aspirating beads. If beads are seen in the pipette tip, dispense the liquid gently, wait for 2 min and repeat the process.

Pause point: DNA can be stored at -80°C for at least six months.

63. Assess the yield of the amplified complementary DNA using fluorometry instrument and assess the DNA profile using capillary automated electrophoresis.

Preparation of DNA library for high-throughput sequencing

Timing: [1 h]

These steps will describe the production of DNA library for next generation sequencing based on bead-linked transposome technology (Bruinsma et al., 2018; Caruccio, 2011) to produce fragments of DNA within a narrow range around 450 base pairs long.

64. Assess the yield of the amplified complementary DNA using fluorometry instrument (e.g., Qubit) and assess the DNA profile using capillary automated electrophoresis (e.g., Bioanalyzer).
65. Prepare a diluted sample of amplified cDNA at 1 ng in 30 μL of water.
66. Follow the protocol described in the Nextera DNA flex Library preparation kit reference guide until the elution of the cleaned library.

Note: Libraries are barcoded in one the specific steps of library construction. In this kit, during the PCR amplification. In our laboratory we use the dual barcode scheme.

Note: Do not carry out the normalization steps for analysis of gene expression.

Note: Carry out 12 cycles of PCR.

67. Assess the yield of the amplified complementary DNA using fluorometry instrument (e.g., Qubit) and assess the DNA profile using capillary automated electrophoresis (e.g., Bioanalyzer). The automated electrophoresis will also be important for the estimation of average fragment length of the library

Alternatives: The data presented in this protocol was prepared with the Nextera DNA flex Library preparation kit, however alternatives to this protocol exist. Since the production of the representative data, Illumina Inc. has released another kit for library production based on the transposon technology. It is also possible to quantify the library DNA using quantitative PCR reagents and the appropriate analytical procedures (Hawkins and Guest, 2018).

Pooling of libraries for high-throughput sequencing

68. Calculate the molarity of each library in nanomolar using the standard equation: $\frac{(concentration\ (ng/\mu l))}{\left(660\frac{g}{mol} \times average\ library\ fragment\ length\ (bp)\right)} \times 10^6$
69. Combine samples maintaining equimolar quantities of material from each library.
70. Submit samples for sequencing according to the sequencing facility's guidelines.

Basic bioinformatic procedures

The processing of the RNA-sequencing data often follows a basic flow of alignment of the reads to the reference genome and counting of the reads according to gene annotation, but there are many variations of this workflow (Conesa et al., 2016; Van den Berge et al., 2019). To produce the representative results, sequences were aligned with Hisat2 (Kim et al., 2015, 2019; Pertea et al., 2016), followed by filtering with samtools (Li et al., 2009) (remove unmapped reads, secondary alignments, failing of platform quality checks, PCR or optical duplicate) and removal of duplicates with the function 'bammarkduplicates' from biobambam (Tischler and Leonard, 2014). Sorting and indexing were done with Picard (http://broadinstitute.github.io/picard/). Finally, fragments were counted with 'featurecounts' (Liao et al., 2014) using the Ensembl (Flicek et al., 2014; Kinsella et al., 2011) bovine annotation as a guide.

The code used to produce representative results is described below.

71. Pipeline for processing raw reads.
    a. Align the reads to the reference genome.

```
#!/bin/bash

##Paths:

-

path_to_index= .../Bos_taurus.ARS-UCD1.2.99

path_to_gtf=.../Bos_taurus.ARS-UCD1.2.98.gtf

splice_sites=.../hisat_splice.txt

path_to_picard=.../bioinfo

merged_fastq=.../merged_fastq

sample=70o

output_alignment= .../alignment/$sample

mkdir $output_alignment

read1=$merged_fastq/sample_51_R1_001.fastq.gz

read2=$merged_fastq/sample_51_R2_001.fastq.gz

/home/fbiase/bioinfo/hisat2-2.2.0/hisat2 -p 30 -k 1 -x $path_to

index -1 $read1 -2 $read2 –known-splicesite-infile $splice_sit

es –no-mixed -S $output_alignment/$sample.alignment.sam –sum

mary-file $output_alignment/$sample.summary.txt

sample=72o

output_alignment= .../alignment/$sample

mkdir $output_alignment

read1=$merged_fastq/sample_55_R1_001.fastq.gz

read2=$merged_fastq/sample_55_R2_001.fastq.gz

/home/fbiase/bioinfo/hisat2-2.2.0/hisat2 -p 30 -k 1 -x $path_to

index -1 $read1 -2 $read2 –known-splicesit

e-infile $splice_sites –no-mixed -S $output_alignment/$sample.alignment.sam –sum

mary-file $output_alignment/$sample.summary.txt

sample=87o

output_alignment= .../alignment/$sample

mkdir $output_alignment

read1=$merged_fastq/sample_67_R1_001.fastq.gz

read2=$merged_fastq/sample_67_R2_001.fastq.gz

/home/fbiase/bioinfo/hisat2-2.2.0/hisat2 -p 30 -k 1 -x $path_to

index -1 $read1 -2 $read2 –known-splicesite-infile $splice_sit

es –no-mixed -S $output_alignment/$sample.alignment.sam –sum

mary-file $output_alignment/$sample.summary.txt

sample=89o

output_alignment= .../alignment/$sample

mkdir $output_alignment
```

```
read1=$merged_fastq/sample_71_R1_001.fastq.gz
read2=$merged_fastq/sample_71_R2_001.fastq.gz
/home/fbiase/bioinfo/hisat2-2.2.0/hisat2 -p 30 -k 1 -x $path_to
_index -1 $read1 -2 $read2 –known-splicesite-infile $splice_sit
es –no-mixed -S $output_alignment/$sample.alignment.sam –sum
mary-file $output_alignment/$sample.summary.txt
sample=91o
output_alignment= .../alignment/$sample
mkdir $output_alignment
read1=$merged_fastq/sample_75_R1_001.fastq.gz
read2=$merged_fastq/sample_75_R2_001.fastq.gz
/home/fbiase/bioinfo/hisat2-2.2.0/hisat2 -p 30 -k 1 -x $path_to
_index -1 $read1 -2 $read2 –known-splicesite-infile $splice_sit
es –no-mixed -S $output_alignment/$sample.alignment.sam –sum
mary-file $output_alignment/$sample.summary.txt
```

b. Filter low-quality alignments.

```
Folder= .../alignment
samtools view -b -h -F 1796 $folder/70o/70o.alignment.sam -o $folder
/70o/70o.alignment.filtered.bam
samtools view -b -h -F 1796 $folder/89o/89o.alignment.sam -o $folder
/89o/89o.alignment.filtered.bam
samtools view -b -h -F 1796 $folder/87o/87o.alignment.sam -o $folder
/87o/87o.alignment.filtered.bam
samtools view -b -h -F 1796 $folder/72o/72o.alignment.sam -o $folder
/72o/72o.alignment.filtered.bam
samtools view -b -h -F 1796 $folder/91o/91o.alignment.sam -o $folder
/91o/91o.alignment.filtered.bam
```

c. Sort alignments by coordinate.

```
java -jar $path_to_picard/picard.jar SortSam INPUT=$folder
/70o/70o.alignment.filtered.bam OUTPUT=$folder/70o/70o.align
ment.filtered.sorted.bam SORT_ORDER=coordinate
java -jar $path_to_picard/picard.jar SortSam INPUT=$folder/
89o/89o.alignment.filtered.bam OUTPUT=$folder/89o/89o.align
ment.filtered.sorted.bam SORT_ORDER=coordinate
java -jar $path_to_picard/picard.jar SortSam INPUT=$folder/
```

```
87o/87o.alignment.filtered.bam OUTPUT=$folder/87o/87o.align
ment.filtered.sorted.bam SORT_ORDER=coordinate
java -jar $path_to_picard/picard.jar SortSam INPUT=$folder
/72o/72o.alignment.filtered.bam OUTPUT=$folder/72o/72o.align
ment.filtered.sorted.bam SORT_ORDER=coordinate
java -jar $path_to_picard/picard.jar SortSam INPUT=$folder
/91o/91o.alignment.filtered.bam OUTPUT=$folder/91o/91o.align
ment.filtered.sorted.bam SORT_ORDER=coordinate
```

d. Index and create index file.

```
java -jar $path_to_picard/picard.jar BuildBamIndex INPUT=$folder
/70o/70o.alignment.filtered.sorted.bam
java -jar $path_to_picard/picard.jar BuildBamIndex INPUT=$folder
/89o/89o.alignment.filtered.sorted.bam
java -jar $path_to_picard/picard.jar BuildBamIndex INPUT=$folder
/87o/87o.alignment.filtered.sorted.bam
java -jar $path_to_picard/picard.jar BuildBamIndex INPUT=$folder
/72o/72o.alignment.filtered.sorted.bam
java -jar $path_to_picard/picard.jar BuildBamIndex INPUT=$folder
/91o/91o.alignment.filtered.sorted.bam
```

e. Remove duplicates.

```
bammarkduplicates I=$folder/70o/70o.alignment.filtered.sorted.bam
O=$folder/70o/70o.alignment.filtered.sorted.undup.bam level=9
markthreads=5 rmdup=1 index=1 dupindex=0 verbose=0
bammarkduplicates I=$folder/89o/89o.alignment.filtered.sorted.bam
O=$folder/89o/89o.alignment.filtered.sorted.undup.bam level=9
markthreads=5 rmdup=1 index=1 dupindex=0 verbose=0
bammarkduplicates I=$folder/87o/87o.alignment.filtered.sorted.bam
O=$folder/87o/87o.alignment.filtered.sorted.undup.bam level=9
markthreads=5 rmdup=1 index=1 dupindex=0 verbose=0
bammarkduplicates I=$folder/72o/72o.alignment.filtered.sorted.bam
O=$folder/72o/72o.alignment.filtered.sorted.undup.bam level=9
markthreads=5 rmdup=1 index=1 dupindex=0 verbose=0
bammarkduplicates I=$folder/91o/91o.alignment.filtered.sorted.bam
O=$folder/91o/91o.alignment.filtered.sorted.undup.bam level=9
markthreads=5 rmdup=1 index=1 dupindex=0 verbose=0
```

f. Count fragments relative to genome annotation.

```
path_to_gtf=.../Bos_taurus.ARS-UCD1.2.98.gtf
folder=.../alignment
folder1=.../counting
/subread-2.0.0-Linux-x86_64/bin/featureCounts -s 0 -a $pa
th_to_gtf -o $folder1/70o/70o.count -F 'GTF' -t 'exon' -g
'gene_id' -ignoreDup -p -T 5 $folder/70o/70o.alignment
.filtered.sorted.undup.bam
/subread-2.0.0-Linux-x86_64/bin/featureCounts -s 0 -a $pa
th_to_gtf -o $folder1/89o/89o.count -F 'GTF' -t 'exon' -g
'gene_id' -ignoreDup -p -T 5 $folder/89o/89o.alignment
.filtered.sorted.undup.bam
/subread-2.0.0-Linux-x86_64/bin/featureCounts -s 0 -a $pa
th_to_gtf -o $folder1/87o/87o.count -F 'GTF' -t 'exon' -g
'gene_id' -ignoreDup -p -T 5 $folder/87o/87o.alignment
.filtered.sorted.undup.bam
/subread-2.0.0-Linux-x86_64/bin/featureCounts -s 0 -a $pa
th_to_gtf -o $folder1/72o/72o.count -F 'GTF' -t 'exon' -g
'gene_id' -ignoreDup -p -T 5 $folder/72o/72o.alignment
.filtered.sorted.undup.bam
/subread-2.0.0-Linux-x86_64/bin/featureCounts -s 0 -a $pa
th_to_gtf -o $folder1/91o/91o.count -F 'GTF' -t 'exon' -g
'gene_id' -ignoreDup -p -T 5 $folder/91o/91o.alignment
.filtered.sorted.undup.bam
```

72. In R software, combine the counts from all files into one matrix.

```
files<-list.files("/mnt/storage/lab_folder/oocyte_project/oocyte_
CC_BCB/counting_JOVE_paper", recursive=T, pattern=".count", full.
names = TRUE)
files<-files[grep("summary", files, invert = TRUE)]
length(files)
count_data<-data.frame(matrix(nrow=27607))
for (n in 1:length(files)) {
  count<-read.delim(files[n], header=TRUE, sep= "\t", stringsAsFa
ctors = FALSE, comment.char= "#")
  count<-count[,c(1,7)]
```

```
  count_data<-cbind(count_data,count)
}
rownames(count_data)<-count_data[,2]
count_data<-count_data[,seq(from = 3, to = 11, by = 2)]
colnames(count_data)<- paste("oocyte" , substr(colnames(count_dat
a), 80, 81), sep="")
```

73. Subset the data to retain genes with > 50 reads

```
count_data_a<-count_data[rowSums(count_data)>50,]
annotation.ensembl.symbol<-read.delim("/resources/2020_05_29
_annotation.ensembl.symbol.txt.bz2", header=TRUE, sep= "\t",
row.names=1, stringsAsFactors = FALSE)
count_data_annotated<-merge(count_data_a,annotation.ensembl.
symbol, by.x="row.names", by.y="ensembl_gene_id", all.x=TRUE,
all.y=FALSE)
count_data_annotated<-count_data_annota
ted[count_data_annotated$gene_biotype %in% c('protein_coding', 'lncRNA','pseudoge
ne'),]
count_data_annotated_length<-count_data_annotated$transcript_length
```

74. Load the necessary libraries

```
libPaths("/usr/lib/R/site-library")
library('dplyr', lib.loc="/usr/lib/R/site-library")
library('ggplot2', lib.loc="/usr/lib/R/site-library")
library('edgeR', lib.loc="/usr/lib/R/site-library")
library("cowplot", lib.loc="/usr/lib/R/site-library")
library("GGally",lib.loc="/usr/lib/R/site-library")
library("DESeq2",lib.loc="/usr/lib/R/site-library")
```

a. Calculate fragments per kilobase and counts per million

```
oocyte_fpkm<-rpkm(count_data_annotated[,c(2:6)], count_data_annotated_length)
oocyte_cpm<-edgeR::cpm(count_data_annotated[,c(2:6)])
```

b. Code to generate Figure 4.

```
table_N_genes_oocyte<-data.frame()
     for (i in seq(0.1,1,0.1)){
       for (j in seq(1,5,1)){
```

```
        oocyte_fpkm_a<-oocyte_fpkm[rowSums(oocyte_fp
km >= i) >= j,]
        dim(oocyte_fpkm_a)[1]
        table_N_genes_oocyte<-rbind(table_N_genes_oo
cyte, data.frame("treshold"=i,"n_samples"=j, "n_genes"=di
m(oocyte_fpkm_a)[1]))
      }
    }
font_size<-12
plot2<-ggplot(table_N_genes_oocyte, aes(treshold, n_genes,
color = n_samples)) +
geom_point() +
scale_x_continuous("FPKM",breaks = seq(0,1,0.1))+
scale_y_continuous("Number of genes \n equal or above FPK
M threshold", breaks = seq(11000,15000,500), limits=c(110
00,15000))+
scale_color_continuous(name = "Sample (N)",breaks = seq(1,
5,1) ,high = "#132B43", low = "#56B1F7")+
guides(colour = guide_legend(reverse=T))+
theme_bw(base_size = font_size)+
theme(panel.grid.major = element_blank(),
   #panel.grid.minor = element_blank(),
   panel.background = element_blank(),
   axis.title=element_text(color="black"),
   axis.text=element_text(color="black"),
   legend.position = c(0.1, 0.25),
   legend.key.size = unit(.1, "cm"),
   legend.text = element_text(size=10),
   legend.title = element_text(size=10))
table_N_genes_oocyte<-data.frame()
    for (i in seq(0.1,1,0.1)){
      for (j in seq(1,5,1)){
        oocyte_cpm_a<-oocyte_cpm[rowSums(oocyte_cpm
>= i) >= j,]
        dim(oocyte_cpm_a)[1]
        table_N_genes_oocyte<-rbind(table_N_genes_oo
cyte, data.frame("treshold"=i,"n_samples"=j, "n_genes"=di
m(oocyte_cpm_a)[1]))
```

```
      }
    }
plot3<-ggplot(table_N_genes_oocyte, aes(treshold, n_genes,
color = n_samples)) +
geom_point() +
scale_x_continuous("CPM",breaks = seq(0,1,0.1))+
scale_y_continuous("Number of genes \n equal or above CPM
threshold", breaks = seq(12000,15000,500), limits=c(1200
0,15000))+
scale_color_continuous(name = "Sample (N)",breaks = seq(1,
5,1) ,high = "#132B43", low = "#56B1F7")+
guides(colour = guide_legend(reverse=T))+
theme_bw(base_size = font_size)+
theme(panel.grid.major = element_blank(),
   #panel.grid.minor = element_blank(),
   panel.background = element_blank(),
   axis.title=element_text(color="black"),
   axis.text=element_text(color="black"),
   legend.position = c(0.1, 0.25),
   legend.key.size = unit(.1, "cm"),
   legend.text = element_text(size=10),
   legend.title = element_text(size=10))
plot_grid(plot2,plot3, nrow=2)
```

c. Transform the counts using the rlog approach.

```
oocyte_count_b<-count_data_annotated[,c(2:6)]
colnames(oocyte_count_b)<-c("oocyte_A", "oocyte_B" ,"oocyte_C" ,"oocyte_D", "o
ocyte_E")
deseq2_oocyte<-DESeqDataSetFromMatrix(oocyte_count_b, colData=DataFrame(c("ooc
yte_A", "oocyte_B" ,"oocyte_C" ,"oocyte_D", "oocyte_E")), design = ~1 )
deseq2_oocyte_RLOG<-rlog(deseq2_oocyte, blind=TRUE)
```

d. Code to generate Figure 5

```
ggpairs(as.data.frame(assay(deseq2_oocyte_RLOG)), xlab = "Regularized Log2 tra
nsformed counts",ylab = "Regularized Log2 transformed counts", diag=list(conti
nuous='blank'), lower = list(continuous = wrap("smooth", alpha = 0.3, size=0.
5)))
```

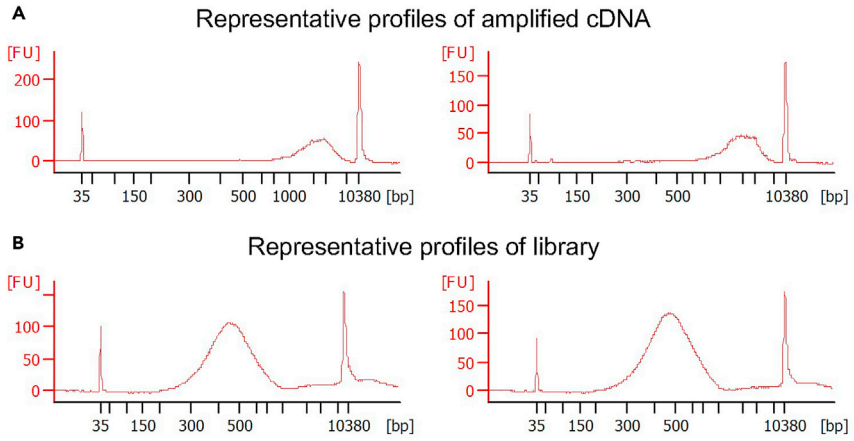

Figure 3. Representative electrophoretic profiles of the DNA produced from two samples using this protocol
(A and B) (A) Amplified complementary DNA, and (B) Amplified DNA containing the insert and adapters for sequencing. The x-axis shows base pairs (bp), and the y-axis shows fluorescence units (FU).

EXPECTED OUTCOMES

The protocol described here was used to produce RNA sequencing data from single bovine oocytes. The wet lab protocol consists of four main procedures: 1- RNA extraction; 2- reverse transcription; 3 - amplification of complementary DNA; and 4- library preparation. Following sequencing assay, the process generally involves, 1-the alignment to the genome; 2- filtering of poor-quality alignment and 3- counting of the reads for quantification of gene transcript abundance (Figure 1A).

Using the protocol described for RNA extraction, the RNA can be indirectly assessed by the amplification of complementary DNA. On average, eight cycles of PCR produce 4.4 ng of amplified complementary DNA (0.44 $\pm$ 0.2 ng/μL, n = 26). The amplification is very homogeneous across cells. Fragment length often averages 2600 nucleotides long, ranging from 700 to ~8000 nucleotides long (Figure 3A).

The amplification of complementary DNA often yields material for multiple library preparations, if necessary. After library preparation, the amplified fragments often range from 250 - 1000 nucleotides long, averaging 457 nucleotides long. On average, ~1 μg of library (37.9 $\pm$ 13.7 ng/μL, n = 26) is produced (Figure 3B).

QUANTIFICATION AND STATISTICAL ANALYSIS

The sequencing of five representative single oocytes collected at the GV stage yielded an average depth of 18,629,853 $\pm$ 1,713,461 non-duplicated pair of reads mapped to the bovine genome built ARS-UCD1.2 (Elsik et al., 2009) at one location and assigned to the bovine Ensembl gene annotation (Flicek et al., 2014; Kinsella et al., 2011). In total, 99.19% of the fragments mapped to protein-coding genes. With simple filtering of the genes that have at least 50 fragments across all five cells, it is possible to identify the detection of 14,204 protein-coding genes, 396 long non-coding RNA, and 82 pseudogenes, which will vary based on the different criterion of filtering and quantification metric (Figure 4). Following data normalization, the average correlation across the gene quantification was 0.99 between samples ($p<0.001$, Figure 5).

LIMITATIONS

Sensitivity is one of the limitations of single-cell RNA-sequencing. The detection of up to 14,204 protein-coding genes presented here is similar to other results from single cattle oocytes obtained with different procedures (Biase and Kimble, 2018; Reyes et al., 2015). It is also very similar to the 14,647 genes detected from a compilation of bulk data from cattle oocytes (Walker and Biase, 2020). Even

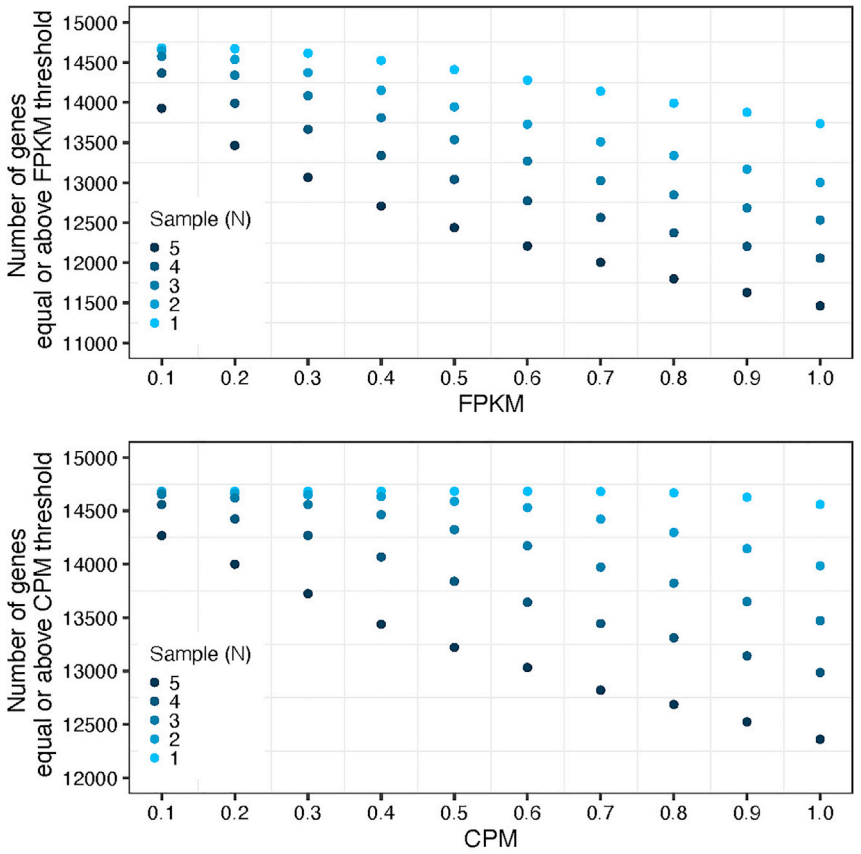

Figure 4. The number of genes detected based on fragments per kilobase per million (FPKM) or counts per million (CPM)

when applying a stringent threshold based on the number of samples and expression levels, it was possible to detect > 11,000 genes. Thus, the protocol presented here detects a large majority of transcripts present in single oocytes, but not necessarily all transcripts.

Technical reproducibility is a serious concern of procedures involving single-cell RNA-sequencing (Stegle et al., 2015). While automation of this protocol is a possibility for somatic cells and spermatozoids, it is not an easy alternative for oocytes. First, obtaining cumulus-oocyte complexes followed by the removal of the cumulus from the oocytes are not trivial tasks that can be executed in hundreds of oocytes at a time. Oocytes must be manipulated carefully and placed in a microtube, which must be immediately snap-frozen. Practicing in several samples prior to working with the research samples should reduce the introduction of technical variability.

Non-polyadenylated (poly(A)-) transcripts are not quantified. Approximately ~1% of the protein-coding genes in the mammalian genome produce poly(A)- transcripts, among which, genes in the histone family are classic examples (Yang et al., 2011; Zhang et al., 2014). Alternative approaches for producing sequencing libraries use random priming and oligo-dT for reverse transcription have been developed (Fang and Akinci-Tolun, 2016), but their efficacy for producing transcriptome from single-cells remain uncertain. This protocol is effective in producing transcriptome data from poly(A)+ transcripts from single oocytes.

TROUBLESHOOTING

Problem 1

Contamination of the reactions.

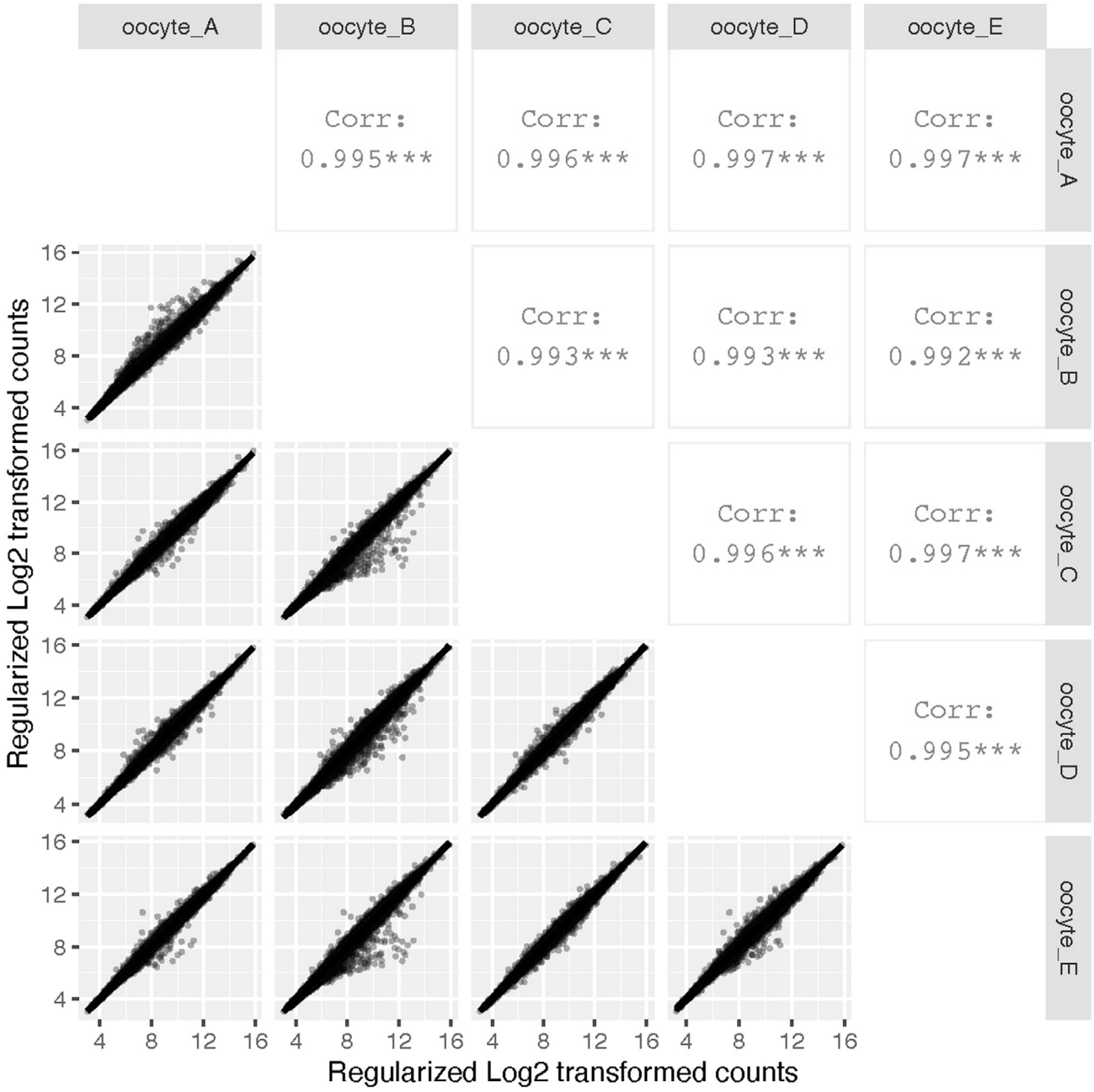

Figure 5. Pair-wise sample correlation of five single-cell RNA sequencing produced from oocytes

Potential solution

It is common for primer dimer and excess of oligonucleotides to be present in non-template negative control. However, should high molecular weight DNA appear in the electrophoresis, all reagents should be replaced with unused reagents to prevent contamination of samples.

Problem 2

No oocyte is deposited in the tube.

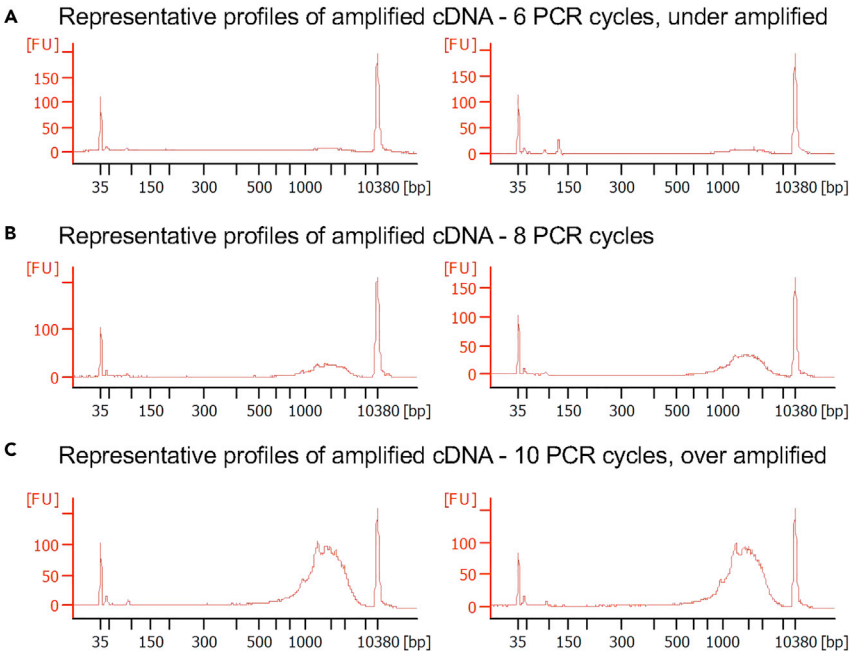

Figure 6. Examples of profiles of amplified complimentary DNA (acDNA)

(A) Examples of acDNA that are not over amplified but did not produce sufficient template for the library preparation.

(B) Examples of acDNA that are not over amplified and produced sufficient template for the library preparation.

(C) Examples of acDNA that are over amplified. Notice the presence of peaks on this profile, which indicate overamplification of specific transcripts.

Potential solution

Researchers will gain experience with the pipetting of small volumes and use of the striper pipette. Diligent and careful pipetting will maximize the chances of depositing the oocyte at the bottom of the tube.

Problem 3

No pellet is produced at the centrifugation with isopropanol.

Potential solution

If there is no pellet at the end of the protocol, it is possible that there was no oocyte deposited in the tube. It is also important that the ratio of sample/Trizol be maintained so that the sample volume does not exceed 10% of the volume of Trizol added to the tube. Refer to step 8.

Problem 4

Under amplification or over amplification of the amplified complementary DNA. Refer to step 49.

Potential solution

Although is it always important to understand that there will be variability of RNA abundance across oocytes, it is important to assess the appropriate number of PCR cycles used for amplification of complementary DNA that will yield a balance between sufficient template for library preparation and high quality amplified complementary DNA in test samples. Please, refer to Figure 6 for examples of suitable amplification and over amplification.

Problem 5

Library has fragments that are broad in range or are outside the expected range.

Potential solution

Set up the tagmentation reaction with tubes on a rack placed on ice. Add the neutralization buffer immediately after the tagmentation reaction is concluded. Refer to step 66.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fernando Biase, fbiase@vt.edu.

Materials availability

This study did not generate new unique reagents.

Data and code availability

The fastq files used as representative outcomes are available from the corresponding author on request. Count data have been deposited to Mendeley Data: http://dx.doi.org/10.17632/fwyk4tfrhg.1. The code used to process raw files, obtain gene transcript abundances, and produce some of the charts presented is presented in steps 71, 72, 73 and 74.

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AUTHOR CONTRIBUTIONS

F.H.B. produced material submitted for sequencing, carried out analysis, and wrote the manuscript.

DECLARATION OF INTERESTS

The author declares no competing interests.

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