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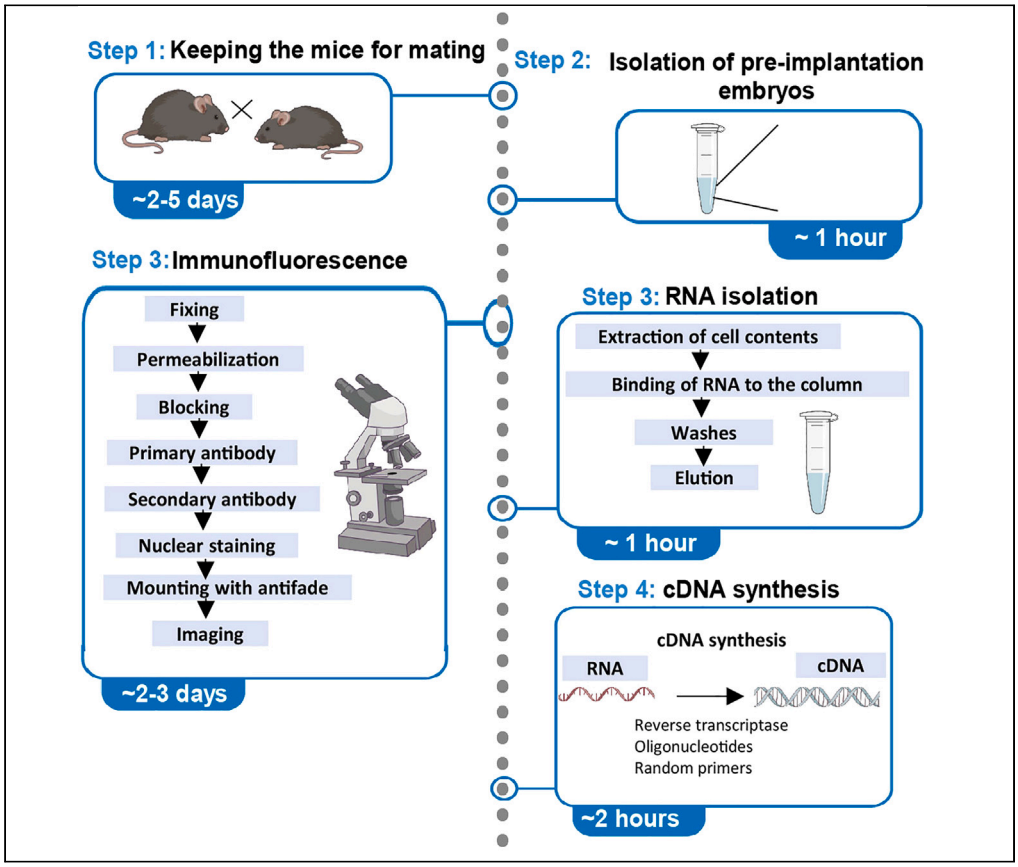
Protocol for isolation of mouse pre-implantation embryos for gene expression analysis



Visualizing and quantifying the numerous factors that regulate murine pre-implantation embryonic development is technically challenging. Here, we present a protocol for the isolation of pre-implantation embryos at multiple stages of embryonic development to study gene expression. We describe steps for isolating RNA and cDNA synthesis from a small number of embryos. We then detail an immunofluorescence assay for the detection and localization of protein of interest by confocal microscopy in the pre-implantation embryos.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Pallavi Chinnu Varghese, Debasree Dutta

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Highlights

The protocol describes isolation of different stages of pre-implantation mouse embryo

Immunofluorescence analysis for expression and localization of protein in mouse embryo

Successful extraction of RNA from 5–10 embryos of different developmental stages

cDNA synthesis from ~50 ng of RNA isolated from embryos, by a commercially available kit

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Protocol for isolation of mouse pre-implantation embryos for gene expression analysis

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SUMMARY

Visualizing and quantifying the numerous factors that regulate murine pre-implantation embryonic development is technically challenging. Here, we present a protocol for the isolation of pre-implantation embryos at multiple stages of embryonic development to study gene expression. We describe steps for isolating RNA and cDNA synthesis from a small number of embryos. We then detail an immunofluorescence assay for the detection and localization of protein of interest by confocal microscopy in the pre-implantation embryos.

For complete details on the use and execution of this protocol, please refer to Varghese et al.[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps for isolating pre-implantation embryos from C57BL/6J mice for immunofluorescence analysis and RNA isolation.

Institutional permissions (if applicable)

All animal experiments should follow the institution specific guidelines. The researchers should receive the permission or ethical clearence from their respective institutions. We conducted the experiments on animals in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines (CPCSEA) and the institutional protocol authorized by the Institutional Animal Ethics Committee (IAEC) (IAEC/678/DSD/2018).

Setting up the animal for mating

Timing: ~40 min

Day-1: 4:00 pm (15 min)

1. Keep 6-weeks to 6 months old female and 2–12 months old male C57Bl/6J mice inside the same cage for mating.

⚠ CRITICAL: The animals should be housed in a cage in a breeding room where a proper temperature, humidity, and 14:10 h light-dark cycle is maintained. Animals should have continuous access to sterile water and food. The age of the female is very important. We have observed 1 and $^1/_2$ month to 2 month old female works best for this particular strain of mice. The age at which the mice reach sexual maturity is 4–6 weeks in females and 6–8 weeks in males. Generally, by 6–8 months some female mouse strains can

become infertile or litter size might reduce. By 10–12 months male mouse can show reduced fertility.[2,3] In mice, age associated decline in fertility arises as a result of reduction in implantation sites or an increased incidence of embryo resorption.[4] This could be attributed to age-associated aneuploidy or level of plasma progesterone during pregnancy.[5,6] Hence, it is important to consider the age of the mice while setting up the mating.

Checking the vaginal plug

Day 2: 8:00 am (15–20 min)

2. Check the female mouse for the presence of a vaginal plug, which indicates mating.
   a. Place the mouse onto the grid cage top and hold the tail of the mouse mid-length with your thumb and index finger.
      i. When the animal extends forward, press the back of the mouse gently with the third and fourth fingers.
      ii. Lift the rear legs up to expose the genitalia.
3. If a plug is observed, that day is considered as 0.5-day post-coitum (dpc). Sometimes the plugs may be difficult to observe.
   a. To ensure the correct observation, use a blunt probe to check for the plug.
   b. If you observe the plug, separate the female mouse into a new cage.

⚠ CRITICAL: Plug checking should be done in the morning time itself, otherwise there is a chance that the plug might fall off or dissolve with time.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (Dilution- 1:200)	Invitrogen, Thermo Fisher Scientific	Cat# A11004
CDH1 (Dilution- 1:100)	Abcam	Cat# ab76055
Biological samples		
Mouse pre-implantation embryos	C57Bl/6J females	1.75dpc (4-cell), 2.25dpc (8-cell), 2.75dpc (morula), 3.5dpc (blastocyst)
Chemicals, peptides, and recombinant proteins		
DMEM	Invitrogen	Cat# AL007A
FBS	Invitrogen, Thermo Fisher Scientific	Cat# 1600044
NaCl	Sigma-Aldrich	Cat# S3014
KCl	Sigma-Aldrich	Cat# P9333
$Na_2HPO_4.2H_2O$	Emplura, Merck	Cat# 1.93622.0521
KH_2PO_4	Rankem	Cat# P0320
Paraformaldehyde	Sigma-Aldrich	Cat# P6148
Triton X-100	Sigma-Aldrich	Cat# T9284
Goat serum	Jackson ImmunoResearch	Cat# 005-00-121
ProLong Gold antifade reagent	Invitrogen, Thermo Fisher Scientific	Cat# P36934
Hoechst 33258 (Used at a concentration of 1 μg/mL)	Sigma-Aldrich	Cat# B1155
Critical commercial assays		
Arcturus Pico Pure RNA isolation Kit	Applied Biosystems, Thermo Fisher	Cat# KIT0204
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	Cat# 4368814

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
*Power*SYBR™ Green PCR Master Mix	Applied Biosystems	Cat# 4367659
Experimental models: Organisms/strains		
C57BL/6J female and male mice, 6-weeks to 6 months old aged females and 2–12 months old aged males	The Jackson Laboratory	https://www.jax.org/strain/000664
Oligonucleotides		
Glyceraldehyde phosphate dehydrogenase (*Gapdh*)	Eurofins	Forward-TGCCCCCATGTTTGTGATG Reverse-TGTGGTCATGAGCCCTTCC
Aprataxin PNK-like Factor (*Aplf*)	Eurofins	Forward- CAAGGAAGCCCTGAAATAACC Reverse-CTGAAAGCTCTGCATTCACCT
Software and algorithms		
NIS	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-confocal
ImageJ	NIH	https://github.com/imagej/ImageJ
Other		
Nunc 4-well dish	Thermo Fisher Scientific	Cat# 176740
1.5 mL microfuge tubes	Tarsons	Cat# 500010
Nunc 15 mL Conical Sterile Polypropylene Centrifuge Tubes	Thermo Fisher Scientific	Cat# 339650
0.2 mL PCR tubes	Axygen	Cat# 321-02-051
250 mL glass bottle	Borosil^{R}	Cat# 1500021
Table top centrifuge	Eppendorf	Cat# 5425 R
Mini rotary shaker	Remi	Cat# RS-12R
Phase contrast microscope	Olympus	Cat# CKX53
Stereo microscope	Olympus	Cat# SZ51
Fluorescence microscope	Olympus	Cat# IX73
Confocal microscopy	Nikon	Cat# A1R si
Mouth pipette	Sigma/Merck, Flexible silicone rubber nosepiece, hard plastic mouthpiece, and 15 inches of latex tubing	Cat# A5177
Drummond Microcaps	Drummond, Volume 50 µL; OD = 1 mm;	Cat# 1-000-0500
Thermal cycler	Applied Biosystems, SimpliAmp™	Cat# A24812
Blunt probe	Fisher Scientific	Cat# 08-995
Glass slide	HiMedia, White color Frosted One End One side, thickness-1.0–1.2 mm	Cat# CG029
Digital pH meter	Systronics	Cat# MK-VI

MATERIALS AND EQUIPMENT

10× PBS		
Reagent	Final concentration	Amount
NaCl	1.37 M	8 g
KCl	26.8 mM	0.2 g
$Na_2HPO_4.2H_2O$	97.75 mM	1.74 g
KH_2PO_4	17.6 mM	0.24 g
Double distilled water		Up to 100 mL
Total		100 mL

When 10XPBS is diluted to 1×, the pH should be 7.4. If required, adjust the pH with HCl and check using a digital pH meter. For prolonged storage, autoclave the 10× PBS and then store it at 25°C–28°C. You can store this buffer for 4–6 months in a 250 mL glass bottle.

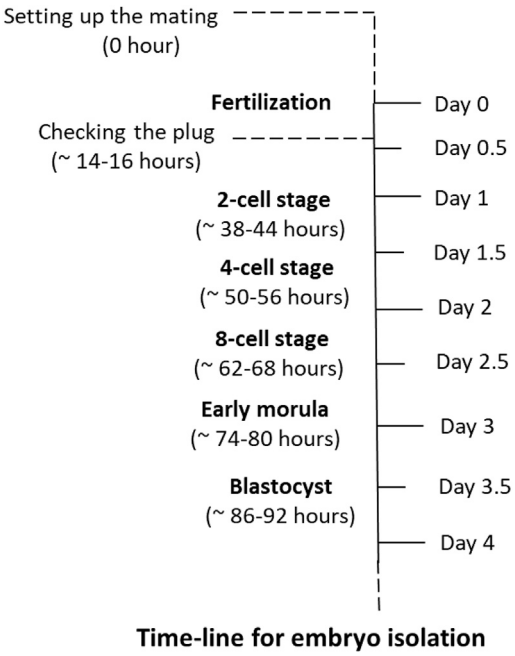

Figure 1. Timeline of murine embryonic development and their isolation

- 4% Paraformaldehyde: add 4 g paraformaldehyde to 1× PBS and make up the volume to 100 mL prepared in a glass bottle. Heat the solution at 55°C in a water bath, until it is completely dissolved. Cool down to 25°C–28°C to be used for immunofluorescence assay or to be stored.

Store at −20°C as aliquots in 15 mL tubes. Wrap the tubes with Aluminum foils for maximum storage of 4–6 months.

- Permeabilization buffer of 0.25% TritonX-100: add 25 µL of TritonX-100 to 1XPBS, make up the volume to 10 mL.

Store at 4°C as aliquots in 1.5 mL microfuge tubes. You can store these tubes for a maximum of 6–12 months.

- Blocking solution: Mix 100% goat serum-1 mL, 0.25% Triton X-100-4 mL and 1× PBS-5 mL.

Store at 4°C as aliquots in 1.5 mL microfuge tubes. You can store these tubes for a maximum of 6–12 months.

⚠ CRITICAL: Paraformaldehyde is a harmful chemical. Gloves should be worn while preparing and using this reagent. Wear face mask to avoid the smell upon heating paraformaldehyde at 55°C.

STEP-BY-STEP METHOD DETAILS

Isolation of pre-implantation embryo

Timing: 1–2 h

The exact time to perform the experiment will depend on which pre-implantation embryo stage you are interested to analyze. A time frame for the isolation of embryos at different stages has been mentioned in Figure 1.[7–11] In addition, the female mice can be superovulated before keeping it

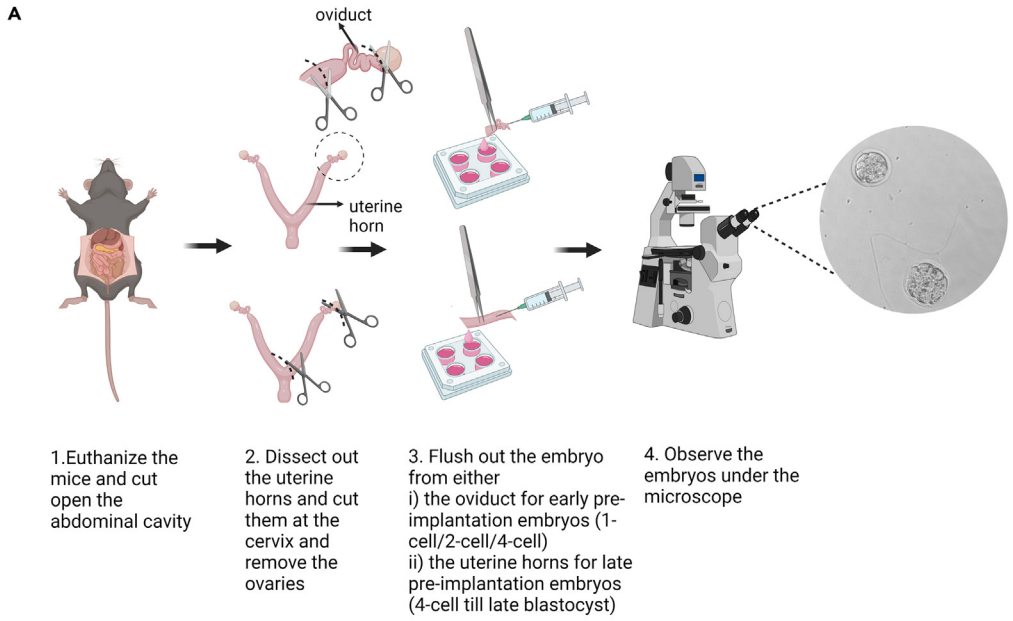

Figure 2. Procedure for the isolation of embryo and usage of mouth pipette

(A) Schematic representation for the isolation of mouse embryo at different stages of development.

(B) Model of mouth pipette used to transfer embryos.

(C) Cartoon representation of holding the mouth pipette while embryo transfer under the microscope.

(D) Closer look into the position of hands while using the mouth pipette.

for mating.[12] This will help to synchronize the developmental stages and increase the yield of embryos at a particular stage.

Using the below mentioned protocol you will be able to isolate pre-implantation embryos. A schematic representation for the isolation of embryos and usage of mouth pipette has been demonstrated in Figure 2.

1. Euthanizing the female mice.
   a. Bring the mice to the euthanasia room and perform cervical dislocation to sacrifice the mice.
2. Dissecting the uterine horn-
   a. Spray alcohol on the belly of the mouse and wipe with tissue paper. Using a tweezer lift up the skin fold and cut open the skin and abdominal lining to expose the uterine horns.
   b. Using fine forceps and scissors cut the uterine horn and place it in a dish containing 1× PBS at 25°C–28°C.
3. Isolation of embryos from the uterine horn.
   a. Cut out the ovaries and also the area adjacent to the joining of the uterine horns.

b. Take media in 1 mL syringe and plunge it into the horns while holding the horns properly with a tweezer such that the media washes out the contents within the fallopian tube into the wells of a 4-well dish.
c. Repeat the process twice for both uterine horns.
d. Allow the embryo to settle down for 10–15 min.
e. Observe the embryos under the phase contrast microscope.
f. Wash the embryos twice or thrice with 1× PBS to remove the debris.
g. Transfer the embryos to the wells of the 4-well plate by mouth pipetting.
h. Take a micrograph with a phase contrast microscope for further reference.

4. Pool the embryos if you are isolating them from multiple female mice and sort the embryos for various procedures.
   a. Proceed to fixation step if the embryo is to be used for immunofluorescence and perform until the blocking stage.
   b. The embryos can be stored at 4°C after blocking, to be analyzed later or can be immediately proceeded for staining.
   c. For RNA isolation, transfer the embryos to a 1.5 mL microfuge tube.
      i. Snap freeze the embryos by immersing the microfuge containing embryos in liquid nitrogen for 2–3 min.
      ii. Store at −80°C.

⚠ CRITICAL: For beginners, sometimes there is confusion in visualizing the embryos. The fat droplets look similar to size of embryos, and the embryos appear to float in different focal planes under the microscope. Hence, it is important to let the embryos settle for 10–15 min before you start looking for them under the microscope. Our experience indicates it is better to start with a phase contrast microscope. When your eyes are set to detect embryos, a stereoscope should be good enough. For your own reference, micrographs should be recorded for each stage of embryonic development. However, if the downstream processing of the embryo involves other studies like the derivation of embryonic or trophoblast stem cells, the transfer of embryos into pseudo-pregnant females, or to study any *in vitro* development of the embryos, a wait time for settling the embryos is not suggested. The embryos in that case should be processed at the earliest.

Immunostaining of mouse pre-implantation embryos

Timing: 2–3 days

All the steps are conducted in shaking condition. The volume of buffers mentioned here is for a batch of embryos.

5. Fix the embryos with 300 μL of 4% paraformaldehyde kept in a single well of a 4-well plate.
6. Incubate at 25°C–28°C for 10 min.

Note: For morula to blastocyst stage embryos, a fixation for 30 min is preferred while 10 min is sufficient for embryos at earlier stages. Depending upon the type of protein to be detected the fixation solution would vary. A freshly prepared fixative should be used. Antibodies that will be used should be checked with proper positive and negative controls. Some antibodies might need more time for fixation. Here, the protein of our interest is E-cadherin and the antibody specific for the protein has been standardized.[1] Also, we have looked into only pre-implantation embryos, hence 4% paraformaldehyde with the mentioned time of incubation is sufficient to fix the embryos.

⚠ CRITICAL: Depending on the quality of the fixation, immunofluorescence analysis for the detection of the protein of interest would differ. Hence, standardization of the fixation protocol with the antibody of your interest is an absolute necessity.

7. Wash the embryos thrice with 300 μL of 1× PBS using mouth pipette and in 4-well plate.
8. Permeabilize the embryo with 300 μL 0.25% TritonX-100, incubate at 25°C–28°C for 1 h.
9. Wash the embryos twice with 300 μL 1× PBS.
10. Incubate the embryos in 300 μL of blocking solution at 25°C–28°C for 2 h or at 4°C for 12–16 h.

Note: The embryos can be stored after blocking at 4°C for several weeks, if you wish to perform the immunostaining later. In fact, we have used embryos almost one year after blocking. In case of prolonged storage, it is better to transfer the embryos into a microfuge with sufficient volume of blocking solution.

Pause point: The embryos processed till now can be stored at 4°C.

11. Dilute primary antibody E-cadherin/CDH1 (1:100) in blocking solution.
12. Transfer embryos to the diluted antibody solution by mouth pipetting.
13. Incubate for 12–16 h at 4°C.

Note: The dilution of the primary antibody will change depending on the isotype, clonality and concentration of the supplied antibody from a company. Hence, the dilution of the primary antibody would vary.

14. The next day, keep the plate at 25°C–28°C for 10 min.
15. Wash the embryos thrice with 300 μL of 1× PBS.

Note: The following steps are light sensitive; hence they should be performed in the dark

16. Add 200 μL of secondary antibody (1:200, diluted with 1× PBS) and keep for 1 h at 25°C–28°C.

Note: After the addition of secondary antibody the embryos may get sticky, so handle them carefully.

17. Wash the embryos thrice with 300 μL of 1× PBS, 10 min each.
18. Add 300 μL of nuclear stain HOECHST (1 mg/mL) diluted in 1× PBS (1:1000), and keep for 10 min at 25°C–28°C.
19. Wash the embryos once with 300 μL of 1× PBS for 5 min at 25°C–28°C.
20. Check the embryos under a fluorescent microscope and carefully transfer them to a slide with a cavity.

Note: A cavity can be made on a normal microscopic slide, as used in this study. Mark a small circle multiple times with a transparent nail polish. The diameter of the circle should be less than the coverslip to be used. However, concave glass slides commercially available (with a shallow depression) can be used to prevent smashing of the embryo by the coverslip.

21. Add a drop of antifade reagent and place the coverslip gently.

Note: Be careful to avoid the formation of bubbles.

22. Fix the coverslip by gently adding transparent nail-polish around the circumference of the coverslip.

23. Keep the slide at 25°C–28°C for 15–30 min.
24. Use confocal microscopy to detect the presence and localization of E-cadherin/CDH1.

Note: You can preserve the slide at 4°C until imaging.

Alternatives: The washing or incubation of the embryos during staining could be done in drops placed in a 60 cm dish, but that might hinder the proper washing of the embryos, for negating any non-specific interaction.

RNA isolation from pre-implantation mouse embryos using Pico-pure kit

Timing: 1 h

This step helps in the isolation of RNA from snap frozen pre-implantation mouse embryos to be used for cDNA preparation and quantitative RT-PCR. We have used Arcturus Pico Pure RNA isolation Kit and have followed the manufacturer's protocol with slight modifications (Applied Biosystems, Thermo Fisher, KIT0204, =%%%https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2F1268200.pdf).

Note: The working area should be properly cleaned. You can turn on the heating block for step 2 before you start. The steps mentioned below are in continuation from the step 4c-II of the section: Isolation of pre-implantation embryo. You can proceed for either immunostaining or RNA extraction after the isolation of the embryos.

25. Add 100 μL of Extraction buffer and resuspend the cell pellet gently by pipetting.
26. Incubate the samples at 42°C for 30 min in a heating block.

Note: You can start conditioning the column by the end of this incubation.

27. Centrifuge the samples at 3000 × *g* for 2 min.
28. Pipette the supernatant containing the extracted RNA into a new microfuge tube.
29. Condition the RNA purification column:
    a. Pipette 250 μL conditioning buffer onto the purification column filter membrane.
    b. Incubate the column with conditioning buffer for 5 min at 25°C–28°C.
    c. Centrifuge the purification column in the provided collection tube at 16000 × *g* for 1 min.
30. Pipette 100 μL of 70% ethanol to the cell extract. Mix well by pipetting up and down. The combined volume must be approximately 200 μL.
31. Pipette the contents into the pre-conditioned purification column.
32. To bind RNA:
    a. Centrifuge the samples for 2 min at 100 g.
    b. Centrifuge once more at 16000 g for 30 s to remove the flow through.
33. Pipette 100 μL wash buffer 1 into purification column and centrifuge for 1 min at 8000 × *g*.
34. Pipette 100 μL wash buffer2 to the purification column and centrifuge for 1 min at 8000 × *g*.
35. Pipette 100 μL wash buffer 2 into the purification column and centrifuge for 2 min at 16000 × *g*.
36. If the wash buffer remains, centrifuge at 16000 × *g* for 1 min.
37. Transfer the purification column to a new 0.5 mL tube supplied with the kit.
38. Pipette the elution buffer directly onto the membrane of the purification column (gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer).

Note: The minimum elution volume to be used is 11 μL, for 6 blastocysts we have used approximately-15 μL, decide accordingly.

39. Wait for 3 min, then spin at 8000 × *g* for 30 s.

40. Discard the column and store the RNA at −80°C.

⚠ CRITICAL: While conducting the entire procedure, you should wear gloves. The amount of RNA isolated from the embryos sometimes might be very little, so we have standardized for how many minimum embryos could be good enough to isolate RNA of good quality that can be used to synthesize cDNA and eventually analyzed for the expression of genes of interest by Sybr Green reporter based quantitative RT-PCR assay. On average 20 embryos per stage (4-cell, 8-cell, morula and blastocyst) are good enough to isolate RNA for cDNA synthesis using the Pico-pure kit.

Pause point: You can store the isolated RNA at −80°C until you are ready to use it.

cDNA synthesis from RNA isolated by Pico-pure kit

Timing: 2 h

41. Measure the RNA concentration by Nanodrop.

Stages	Number of embryos used	Approximate yield of total RNA
4-cell	10	120 ng
8-cell	10	150 ng
Morula	5	120 ng
Blastocyst	5	150 ng

42. Prepare the master mix with the cDNA high capacity reverse transcriptase kit as- 10× RT Buffer-2.0 μL, 25× dNTP Mix (100 mM) 0.8 μL, 10× RT Random Primers 2.0 μL, MultiScribe™ Reverse Transcriptase 1.0 μL, RNase Inhibitor 1.0 μL, Nuclease-free water 3.2 μL with total volume per reaction 10.0 μL (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fcms_042557.pdf).
43. Take at least 50 ng of RNA in a 0.2 mL PCR tube and make up the volume to 10 μL with nuclease free water.
44. Add 10 μL of master mix to the RNA in the PCR tube.
45. Mix by pipetting once or twice, spin for a pulse of 10 s at 800 × *g*.
46. Keep the tube on ice until you start the Thermal Cycler to carry out the PCR.
47. PCR cycling conditions are- 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 4°C hold.
48. Once the reaction is over, store the sample at −20°C.

EXPECTED OUTCOMES

This protocol is used to isolate mouse pre-implantation embryos followed by detection of protein localization by immunostaining and determination of gene expression by RNA isolation and cDNA synthesis. Using the protocol described here, we have successfully isolated 4-cell, 8-cell, morula and blastocyst stages of mouse embryonic development (Figure 3). We have performed immunofluorescence analysis to detect the expression of CDH1 protein in embryo (Figure 4). We successfully extracted RNA from limited number of embryos, followed by cDNA synthesis and analyzed the gene expression of the histone chaperone *Aplf* in this set of embryos (Figure 5).

LIMITATIONS

The embryo number is a constraint for the isolation of sufficient and good quality of RNA especially for the earlier stages of development.

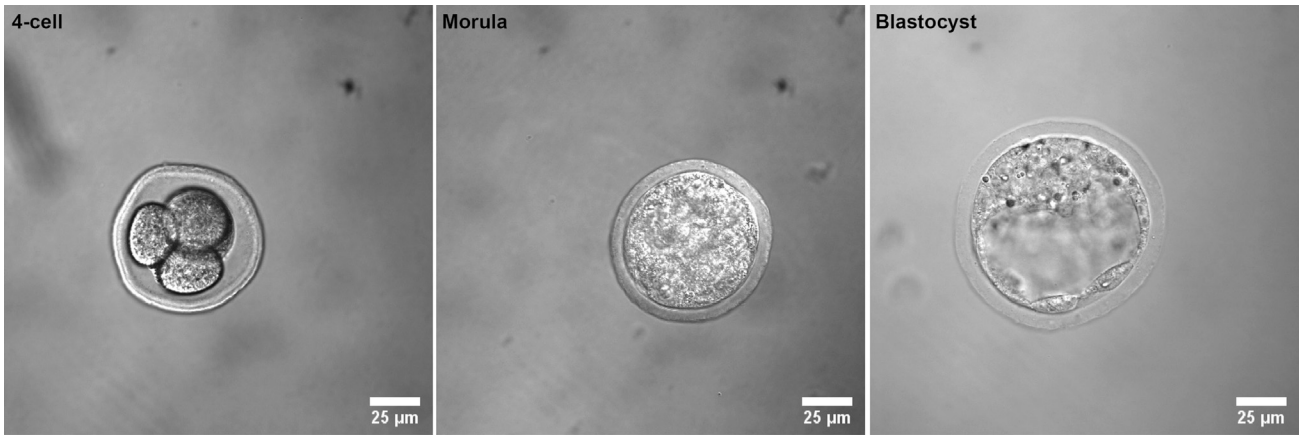

Figure 3. Phase contrast micrograph of embryos isolated at different developmental stages
Scale bar= 25µm, 60X magnification, 1X Zoom.

TROUBLESHOOTING

Problem 1

Checking the vaginal plug- step 3

- Missing the vaginal plug. This will create confusion in counting of the embryonic days leading to mistakes in the isolation of the embryo at a particular stage of development.

Potential solution

- In addition to checking the plug, the vaginal smear can also be collected to check the presence of sperm under the microscope.

Problem 2

Isolation of pre-implantation embryo -step 24

- Manipulating embryos in a 4-well plate. There is every chance of missing them while completing all the steps for immunofluorescence analysis, especially for a beginner.

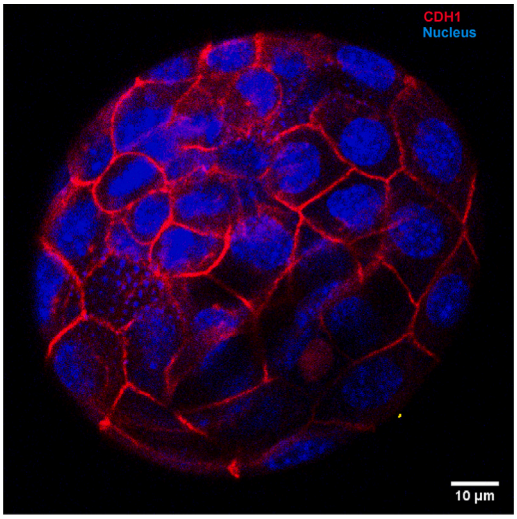

Figure 4. Confocal microscopy image of a E3.5 blastocyst demonstrating the expression of E-cadherin (CDH1)
Scale bar = 10µm, 60X magnification, 3X Zoom.

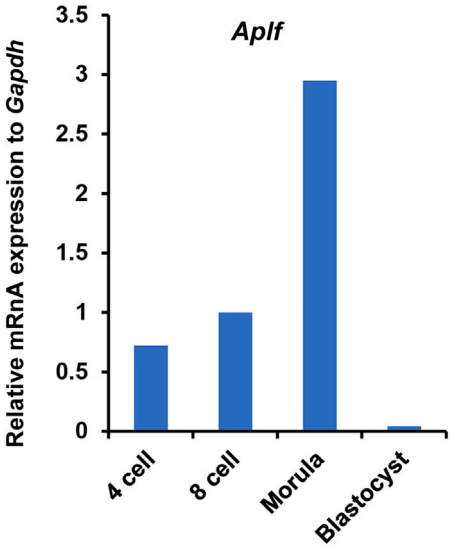

Figure 5. Relative mRNA expression of the histone chaperone *Aplf* determined by quantitative RT-PCR

Potential solution

- Practice the transferring and manipulation of embryo with the mouth pipette in drops of media, at first, before shifting to 4-well plate.

Problem 3

Checking the vaginal plug- step 3

- No observation of a plug or no evidence of mating

Potential solution

- Change the breeder pair. It is advantageous to take a "stud" male which has been successful in mating earlier. Generally use of an older male than the female mouse enhance the mating chances.

Problem 4

Isolation of pre-implantation embryo- step 3

- No embryos were found after dissecting the uterine horn.

Potential solution

- Make sure the uterine horn is cut from both the ends, ovary side and uterus side, quite closely so that no portion of the horn is lost. Also ensure to flush the horns from both the sides.

Problem 5

Immunostaining of mouse pre-implantation embryos – Step 13

- Immunofluorescence analysis shows non-specific expression or a high background noise-to-signal ratio.

Potential solution

- The fixing protocol for staining needs to be standardized. Same antibody from multiple companies might need a change in incubation time with the fixative. Safely, fixative should be prepared fresh.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Debasree Dutta (debasreedutta@rgcb.res.in).

Materials availability

This protocol does not generate any new material.

Data and code availability

Not applicable to this protocol.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

P.C.V. set up the mating, checked the plugs, and isolated the embryos from female mice; P.C.V. and D.D. conducted the immunofluorescence experiments; P.C.V. extracted mRNA from embryos, prepared cDNA, and analyzed gene expression by qRT-PCR; P.C.V. and D.D. prepared figures and wrote and finalized the manuscript; D.D. provided the reagents and equipment required for the protocol to be performed.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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