Video Article

Single Oocyte Bisulfite Mutagenesis

Michelle M. Denomme[1,2,3], Liyue Zhang[3], Mellissa R.W. Mann[1,2,3]

[1]Department of Obstretrics & Gynaecology, Schulich School of Medicine and Dentistry, University of Western Ontario

[2]Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario

[3]Children's Health Research Institute

Correspondence to: Mellissa R.W. Mann at mmann22@uwo.ca

URL: http://www.jove.com/video/4046
DOI: doi:10.3791/4046

Keywords: Genetics, Issue 64, Developmental Biology, Biochemistry, Bisulfite mutagenesis, DNA methylation, individual oocyte, individual embryo, mouse model, PCR, epigenetics

Date Published: 6/27/2012

Citation: Denomme, M.M., Zhang, L., Mann, M.R. Single Oocyte Bisulfite Mutagenesis. *J. Vis. Exp.* (64), e4046, doi:10.3791/4046 (2012).

Abstract

Epigenetics encompasses all heritable and reversible modifications to chromatin that alter gene accessibility, and thus are the primary mechanisms for regulating gene transcription[1]. DNA methylation is an epigenetic modification that acts predominantly as a repressive mark. Through the covalent addition of a methyl group onto cytosines in CpG dinucleotides, it can recruit additional repressive proteins and histone modifications to initiate processes involved in condensing chromatin and silencing genes[2]. DNA methylation is essential for normal development as it plays a critical role in developmental programming, cell differentiation, repression of retroviral elements, X-chromosome inactivation and genomic imprinting.

One of the most powerful methods for DNA methylation analysis is bisulfite mutagenesis. Sodium bisulfite is a DNA mutagen that deaminates cytosines into uracils. Following PCR amplification and sequencing, these conversion events are detected as thymines. Methylated cytosines are protected from deamination and thus remain as cytosines, enabling identification of DNA methylation at the individual nucleotide level[3]. Development of the bisulfite mutagenesis assay has advanced from those originally reported[4-6] towards ones that are more sensitive and reproducible[7]. One key advancement was embedding smaller amounts of DNA in an agarose bead, thereby protecting DNA from the harsh bisulfite treatment[8]. This enabled methylation analysis to be performed on pools of oocytes and blastocyst-stage embryos[9]. The most sophisticated bisulfite mutagenesis protocol to date is for individual blastocyst-stage embryos[10]. However, since blastocysts have on average 64 cells (containing 120-720 pg of genomic DNA), this method is not efficacious for methylation studies on individual oocytes or cleavage-stage embryos.

Taking clues from agarose embedding of minute DNA amounts including oocytes[11], here we present a method whereby oocytes are directly embedded in an agarose and lysis solution bead immediately following retrieval and removal of the zona pellucida from the oocyte. This enables us to bypass the two main challenges of single oocyte bisulfite mutagenesis: protecting a minute amount of DNA from degradation, and subsequent loss during the numerous protocol steps. Importantly, as data are obtained from single oocytes, the issue of PCR bias within pools is eliminated. Furthermore, inadvertent cumulus cell contamination is detectable by this method since any sample with more than one methylation pattern may be excluded from analysis[12]. This protocol provides an improved method for successful and reproducible analyses of DNA methylation at the single-cell level and is ideally suited for individual oocytes as well as cleavage-stage embryos.

Video Link

The video component of this article can be found at http://www.jove.com/video/4046/

Protocol

DAY 1

Prepare the following solutions fresh on the day of oocyte collection with sterile, distilled water such as GIBCO water. To reduce the chance of DNA contamination, change gloves often and use filter tips. Keep tubes angled away when open, and recap all tubes when not in use. We recommend that solutions are made as n+1.

3% LMP Agarose

30 mg Low Melting Point (LMP) Agarose

up to 1 ml GIBCO H_2O

dissolve @ 70 °C

Lysis Solution

8 µl lysis buffer

1 µl proteinase K

1 µl 10% IGEPAL

place on ice until ready for use

2:1 Agarose:Lysis Solution (10 µl per individual oocyte, amount is for 3 oocytes)

20 µl 3% LMP agarose

10 µl Lysis Solution

mix @ 70 °C

SDS Lysis Buffer (501 µl per individual oocyte)

1x TE pH 7.5	450 µl
10% SDS	50 µl
Proteinase K	1 µl
	501 µl

1. Oocyte Collection

1. Place the dissected mouse oviducts in M2 media, and tear the ampullae to extract the cumulus cell complex.
2. Separate the oocytes from the cumulus cell complex using 0.3 mg/ml hyaluronidase solution in a 30 µl drop of M2 media. Keep the oocytes in solution only as long as it takes to remove the cumulus cells, as lengthy exposure may damage them. Wash the oocytes 3x in 30 µl drop of M2 media, removing cumulus cells periodically.
3. Remove the zona pellucida using acidic tyrode's solution. Place the oocytes in one 30 µl drop of solution first, and then transfer to another 30 µl drop, as any media carried along will dilute the acid and reduce its efficiency. Keep the oocytes in solution only as long as it takes to remove the zona, as lengthy exposure may damage them. Note: an increased concentration of acidic tyrode's solution or pronase may be used for human samples, as the human zona pellucida is more resistant to treatment with acidic tyrode's solution than the mouse.
4. Wash the oocytes once more in a 30 µl drop of M2 media.

2. Agarose Embedding and Lysis

1. To perform agarose embedding, place the lysis solution on a 70 °C heatblock. Add the preheated LMP agarose to the lysis solution, producing a 2:1 agarose:lysis solution.
2. Place a single oocyte onto a clean glass slide in minimal M2 media. Take up 10 µl of the agarose:lysis solution into a pipette tip, and (under a microscope) gently expel a small amount (~1 µl or less) onto the glass slide, allowing it to mix with the minimal media. Gently pick up the oocyte into the pipette tip and put all 10 µl into an Eppendorf tube with 300 µl mineral oil so the bead forms a sphere.

   Note: this process must be done fairly quickly as the agarose will harden if the temperature drops as little as 5 °C below 70 °C.
3. Incubate the tube on ice for 10 minutes. To perform lysis, remove the 300 µl mineral oil and add 500 µl of the SDS lysis buffer. Incubate overnight in a 50 °C water bath.

   Note: Lysis solution (**Table 1**) may also be used for this purpose.

DAY 2

Prepare the following solutions fresh on the day of bisulfite mutagenesis. To reduce chance of DNA contamination, change gloves often and use filter tips. Keep tubes angled away when open, and recap all tubes when not in use. We recommend that solutions are made as n+1.

3 M NaOH	2.4 g NaOH in 20 ml autoclaved ddH_2O
0.1 M NaOH	0.5 ml of 3M in 14.5 ml autoclaved ddH_2O
0.3 M NaOH	1.5 ml of 3M in 13.5 ml autoclaved ddH_2O

2.5 M Bisulfite Solution

a. 3.8 g sodium bisulfite

   5.5 ml GIBCO distilled H_2O

   1 ml 3 M NaOH

dissolve @ room temperature

b. 110 mg Hydroquinone

1 ml GIBCO distilled H_2O

dissolve @ 90 °C (for only as long as it takes to dissolve, mix regularly)

When fully dissolved, mix solution (a) and (b)

Keep away from light

3. Bisulfite Mutagenesis

1. Fully remove the 500 µl SDS lysis buffer and add 300 µl mineral oil (~20 hours). Any lysis buffer remaining will dilute the agarose when it is heated and the bead will be more susceptible to dissolving in the subsequent steps. Proceed with bisulfite mutagenesis immediately, or store at -20 °C for up to 5 days.
2. If applicable, remove oocytes from the freezer and let thaw (only until agarose bead is relatively translucent). Incubate for 2.5 minutes on a 90 °C heat block, following which Incubate on ice for 10 minutes.

   Note: Do not mix or stir, extend longer than 2.5 minutes, or fluctuate temperature.
3. To perform denaturation, remove the mineral oil and add 1 ml 0.1M NaOH to each tube, flick and invert 5-6 times.
4. Incubate for 15 minutes in a 37 °C waterbath, inverting every 3-4 minutes. The bead should float in the NaOH.
5. To perform bisulfite treatment, spin the tube gently, then remove the NaOH and add 300 µl mineral oil and 500 µl bisulfite solution. Incubate the tube for 3.5 hours in a 50 °C waterbath. ***Keep away from light***

   Note: Length of incubation may need to be empirically determined for gene of interest.
6. To perform desulfonation, incubate on ice for 3 minutes, then remove the mineral oil and the bisulfite solution, spin gently, and add 1 ml 0.3 M NaOH. Flick and invert 5-6 times.
7. Incubate for 15 minutes in a 37 °C waterbath, inverting every 3-4 minutes. The bead should float in the NaOH.
8. Wash the samples, by first spinning gently, then remove the NaOH and add 1 ml 1x TE pH 7.5. Shake for 5-10 minutes at room temperature (on a shaker). Spin gently again, then remove the 1x TE. Repeat this washing process twice.
9. Add 1 ml autoclaved ddH_2O. Shake for 5-10 minutes at room temperature (on a shaker). Spin gently, then remove the H_2O. Repeat ddH_2O wash twice.
10. Check the pH of the supernatant; it should be pH 5.0. If still too basic, wash again with H_2O. Remove all supernatant, leaving only the agarose bead.

4. 1st and 2nd Round PCR amplification

1. Prepare 1st round PCR mix **while washing**

10 µM Primer Forward Outer	0.5 µl
10 µM Primer Reverse Outer	0.5 µl
240 ng/ml tRNA	1 µl
H_2O	13 µl

Add to Illustra Ready-to-Go Hot Start PCR beads

Carefully slide the solid agarose bead into the PCR tube (~10 µl)

Heat to 70 °C and mix

Add 25 µl mineral oil

Total: **50 µl**

2. Amplify

   Note: An example of cycling conditions for mouse *Snrpn* is denaturation for 2 minutes at 94 °C, followed by 40 cycles of 30 seconds at 94 °C, 1 minute at 50 °C, and 1 minute at 68 °C; and a final 10 minute elongation step at 68 °C. Annealing temperature for 1st round PCR for mouse *H19* and *Peg3* is 50 °C.
3. Prepare 2nd round PCR mix

10 µM Primer Forward Inner	0.5 µl
10 µM Primer Reverse Inner	0.5 µl
H_2O	19 µl

Add to Illustra Ready-to-Go Hot Start PCR beads

Add 5 µl 1st Round product as a template. Heat the 1st round product to 70 °C for 1 minute to soften the agarose. Be sure to pipette below the layer of mineral oil.

Add 25 µl mineral oil

Total: 50 µl

Note: Nested primer sequences for *Snrpn, H19,* and *Peg3* can be found in Market-Velker *et al*[10,12].

4. Amplify

   Note: Cycling conditions for mouse *Snrpn* is denaturation for 2 minutes at 94 °C, followed by 40 cycles of 30 seconds at 94 °C, 1 minute at 50 °C, and 1 minute at 68 °C; and a final 10 minute elongation step at 68 °C[10]. Mouse *H19* and *Peg3* require a 50 °C annealing temperature for 2nd round PCR.
5. As a diagnostic test, second round samples can be cut with a restriction enzyme that is methylation- or strain-specific.

2nd Round product	4 µl
Restriction Enzyme	1 µl
Buffer	1 µl
H_2O	4 µl

6. Electrophorese the digestion products on an 8% acrylamide gel. Any heterogeneous bands represent more than one sequence.

5. TA Cloning and Colony PCR

1. To clone 2nd round product, first heat to 70 °C for 1 minute to soften the agarose, then ligate into vector using the Promega pGEM-T Vector System (Fisher Scientific Cat#A1360).

2nd Round PCR	1 µl
pGEMT-EASY vector	1 µl
Ligase	1 µl
H_2O	2 µl
2x Ligation Buffer	5 µl

Incubate overnight @ 4 °C in PCR machine.

2. Thaw competent *E.coli* cells on ice for 15 minutes (Zymo Research Corp Cat#T3009). Add 3 µl ligation reaction to 8 µl *E.coli* and incubate ligation on ice for 15 minutes.
3. Heat shock for 40 seconds in a 42 °C waterbath, and incubate on ice for 2 minutes. Add 60 µl S.O.C. medium and incubate at 37 °C for 1 hour (in shaker).
4. Place all of the reaction mix on an LB/Agar/IPTG/Xgal/Amp plate and incubate plate at 37 °C overnight.
5. Prepare colony PCR mix

20 µM M13 Forward Primer	0.7 µl
20 µM M13 Reverse Primer	0.7 µl
5X Green Go Taq Buffer	7.0 µl
10 mM dNTP	0.7 µl
Taq DNA polymerase	0.28 µl
H_2O	25.62 µl
	35 µl Total

Add 35 µl Colony PCR master mix into a PCR tube. Pick a white bacterial colony from the plate with a pipette tip, and swirl it into the PCR reaction.

6. Amplify with denaturation for 10 minutes at 94 °C, followed by 30 cycles of 45 seconds at 94 °C, 30 seconds at 57 °C, and 1 minute at 72 °C; and a final 10 minute elongation step at 72 °C. Electrophorese 4 µl on a 1.5% agarose gel. Send ~30 µl of the PCR product for sequencing.

   Note: For oocytes, 5 colony PCR products are sequenced.
7. Once sequencing results are obtained, methylation patterns can be read. Any original CG that remained as a CG was methylated, and any original CG that is now a TG was unmethylated.

6. Representative Results

In our work, we assay imprinted methylation in individual oocytes and embryos **(Figure 1)**. Following nested PCR amplification using bisulfite converted primers, it is possible to confirm a successful conversion by visualizing a correct fragment size on an agarose gel **(Figure 2).** An individual oocyte represents one parental allele, and in theory, has one imprinted methylation pattern. As such, second round PCR products can be tested for unintentional contamination. A restriction enzyme sensitive to DNA methylation (such as *HinfI* or *DpnII*) can be used to digest the second round PCR product to assess whether it contains a methylated or unmethylated allele **(Figure 3)**. A methylated C within the enzyme recognition sequence is cleaved while an unmethylated C that is converted to T is no longer recognized by the enzyme and is uncut. Any MII oocyte sample containing both methylated and unmethylated alleles should be discarded, as it is indicative of cumulus cell contamination **(Figure 3)**. Following ligation and transformation, successful colony PCR amplification can be visualized on an agarose gel to ensure samples with the correct product size are sent for sequencing **(Figure 4)**. Finally, the sequence of five individual clones from an MII oocyte should produce five identical methylation patterns and identical nonCpG conversion rates **(Figure 5a)**. Any samples that contain more than one pattern should be discarded **(Figure 5b)**. Since ovulated MII oocytes have two chromosome copies or an attached polar body, there is a possibility for obtaining two similar sequence patterns **(Figure 5c)**. We recommend discarding data from oocytes that have highly dissimilar methylation patterns since cumulus cell contamination cannot be ruled out.

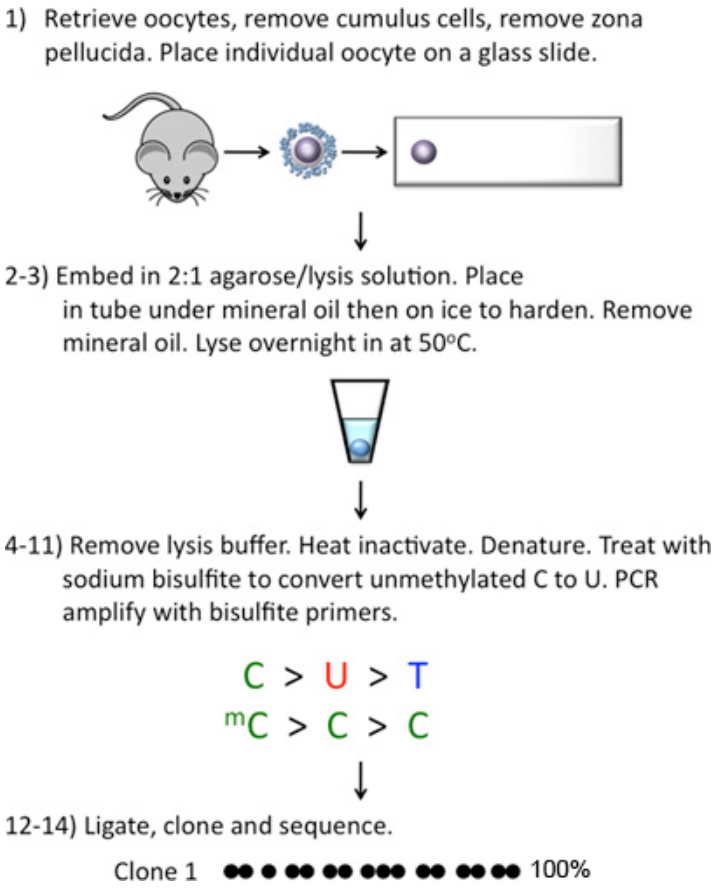

Figure 1. Schematic of the Single Oocyte Bisulfite Mutagenesis assay.

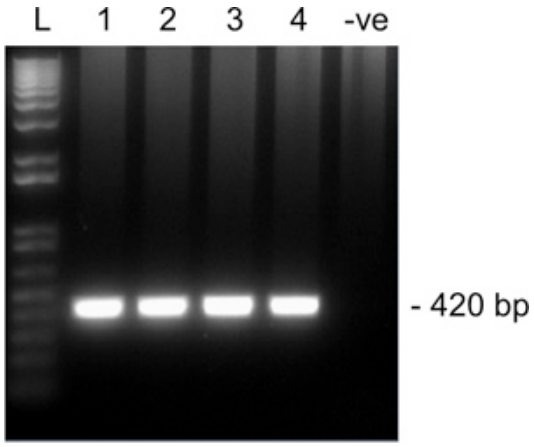

Figure 2. Representative results from 2nd round amplification for *Snrpn* from a single MII oocyte on a 1.5% agarose gel. Lanes 1-4 are four single MII oocytes and lane 5 is a negative control (no oocyte). Expected amplicon size for *Snrpn* is 420 bp. L, ladder.

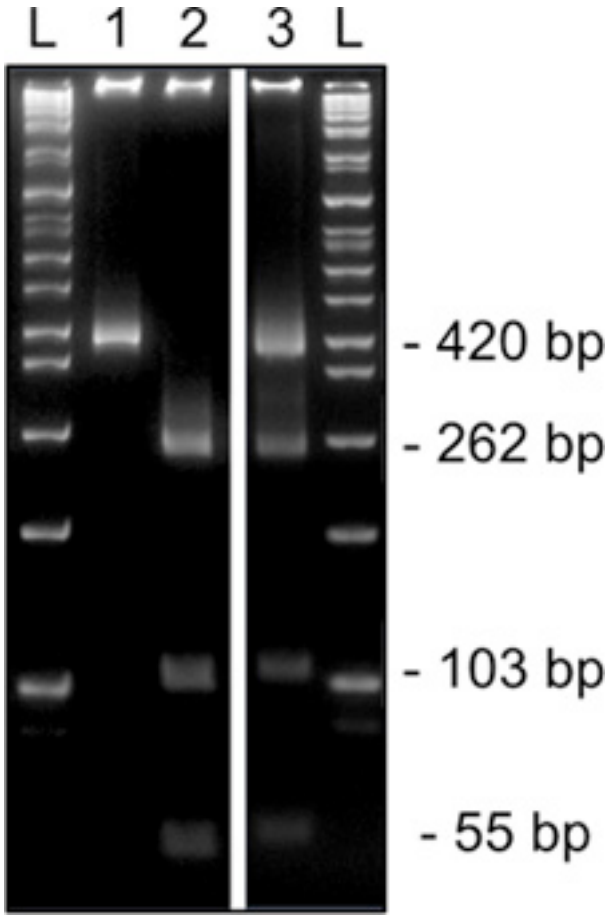

Figure 3. Representative results from 2nd round methylation-specific restriction digestion for *Snrpn* from a single MII oocyte on an 8% acrylamide gel. *HinfI* diagnostic restriction digestion shows unmethylated DNA which harbors a T that abolishes the restriction site (420bp, lane 1) or methylated DNA which contains a C within recognition site (cut, 262, 103, and 54 bp, lane 2). Digestion showing both methylated and unmethylated restriction enzyme sites (cut & uncut bands, lane 3) are indicative of cumulus cell contamination. L, ladder.

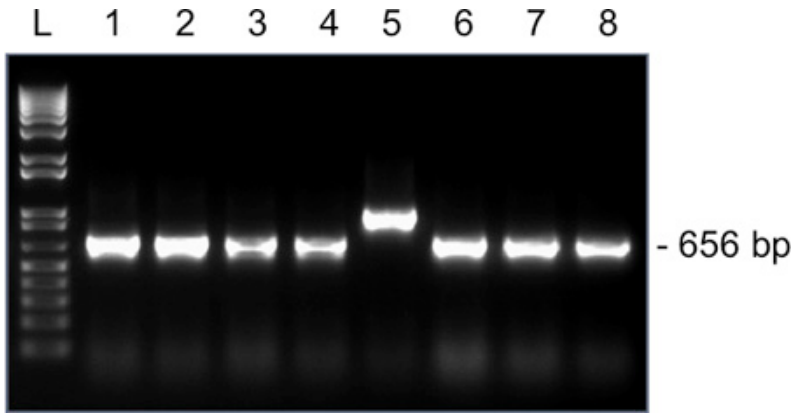

Figure 4. Representative results for colony PCR amplification for *Snrpn* from a single MII oocyte on a 1.5% agarose gel. Expected amplicon size following ligation of *Snrpn* into the pGEM-T Easy vector and using M13 forward and reverse primers is 656 bp. Lane 1-8, amplicons from clones 1-8. Clone 5 has an incorrect amplicon size and should not be sent for sequencing.

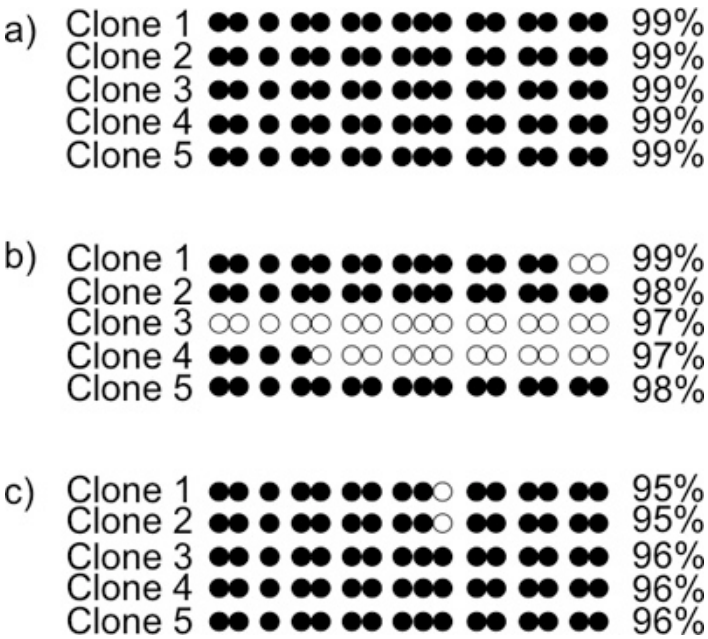

Figure 5. Representative sequencing results for *Snrpn* from a single MII oocyte. *Snrpn* is methylated in oocytes. Black circles indicate methylated CpGs. White circles indicate unmethylated CpGs. CpG number and placement is representative for a B6 strain female mouse. a) Expected sequencing results for *Snrpn* from a single MII oocyte. Only a single strand of DNA should amplify in all five clones. Oocytes with a single methylation pattern and identical non-CpG conversion pattern should be included in analyses (percent conversion of non-CpGs indicated to the right was calculated as the number of non-CpG cytosines converted to thymine as a percentage of total non-CpG cytosines). b) Sequencing results for *Snrpn* from a single MII oocyte with cumulus cell contamination. Note the dissimilarity between methylation states and conversion patterns indicating multiple strand amplification. c) Sequencing results for *Snrpn* from a single MII oocyte with both chromosome copies or polar body inclusion.

Discussion

This single oocyte assay contains many steps with a number that are critical and require special care. The first is oocyte washing. It is particularly important to wash each oocyte multiple times in fresh medium drops following hyaluronidase treatment to remove as many cumulus cells as possible. Moreover, when transferring oocytes to acidic tyrode's solution for zona pellucida removal make sure surrounding medium is clear of cumulus cells. The oocyte is very sticky following zona removal, and any surrounding cumulus cells can easily become stuck to the oocyte. It is very difficult to remove a cumulus cell that is stuck to a zona-free oocyte. While this protocol allows for detection of cumulus cell contamination at later time points, it is not constructive, nor economical, to undergo the full protocol on oocytes that will likely be discarded.

The second critical step is oocyte embedding. When embedding the oocyte in the agarose and lysis solution, it is important to note that low melting point (LMP) agarose will harden at temperatures as little as 5 °C below 70 °C. As such, we recommend mixing the agarose and lysis

solution at 70 °C, and then placing the individual oocyte on the glass slide in minimal medium. Once ready, pipette the agarose/lysis solution and embed the oocyte into a prepared Eppendorf tube under mineral oil, in a careful yet timely manner.

It is critical that heat inactivation of the proteinase K be performed at 90 °C for 2.5 minutes. Deviation from this is not recommended. Higher temperatures or longer times may damage the DNA, while lower temperatures or shorter times may not inactivate the proteinase K.

As a reminder, sodium bisulfite and hydroquinone are light sensitive. Solutions should be prepared and then wrapped in foil, amber tubes and/or placed in a dark drawer until use. Once the bisulfite solution has been added to the tube, the tube should be covered with foil or a dark covered bag when incubating at 50 °C. We use empty foil bags from GE Healthcare that originally contained the hot start PCR beads.

Finally, as with most PCRs, we recommend preparing each round in a timely manner. Excessive delays, particularly after mixing at 70 °C, will reduce success rate of amplification.

At the current time, one limitation of the protocol is that the success rate of bisulfite converted DNA amplification from individual oocytes ranges from 40% to 65% depending on the gene fragment amplified. While additional trouble-shooting may increase this percentage, there is a trade-off between the bisulfite conversion treatment being too long or harsh and not having sufficient conversion rates (>85-90%). A second limitation is that currently bisulfite mutagenesis cannot distinguish between repressive 5-methylcytosine and the demethylation intermediate 5-hydroxymethylcytosine[13].

Several modifications may be required based on gene or region of interest. The bisulfite conversion time may require optimization for the highest conversion percentage with the lowest DNA damage. We suggest a range of 2.5 to 4 hours (half hour increments) for bisulfite treatment. Further optimization can involve PCR primer design for converted sequences of interest (for example http://www.urogene.org/methprimer/), as well as optimization of PCR programs based on fragment length and CG content (see Patterson *et al*. for additional bisulfite mutagenesis optimization options[7]). A final modification is that gel extraction of the 2nd round PCR product may be required if there is abundant primer-dimers or non-specific amplicons that will integrate into the vector when cloned.

We have previously shown that this protocol is effective for individual MII oocytes[12]. We have also tested its efficiency on growing oocytes at a range of different oocyte diameters, with the same rates of successful amplification of converted DNA. Importantly, in comparison to other bisulfite mutagenesis procedures, which are not efficacious for methylation studies on an individual cell, this protocol enables the analysis of DNA methylation of individual oocytes and early embryos. This is essential for reproductive and developmental biology studies, specifically as it relates to the manipulations of germ cells and early embryos. Future applications may include analysis of DNA methylation patterns in single cells of any origin, including *in vivo*-derived and cultured cells. Additionally, we have developed an individual blastocyst assay that recovers DNA and RNA from the same embryo, allowing for both expression and methylation data to be obtained. A future application will involve modifying the single oocyte assay to recover RNA for expression analyses as well as DNA for methylation assays. Another optimization would be to perform duplex assays for the same oocyte, similar to El Hajj *et al*[14], that would allow for methylation detection at a somatically methylated, oocyte-embryo unmethyated gene, thereby permitting detection of cumulus cell contamination.

DNA methylation analyses can range from genome-wide to locus-specific. Genome-wide methods such as methylated DNA immunoprecipitation (MeDIP) in conjunction with microarrays or sequencing typically require abundant amounts of material. Locus-specific methods that include combined bisulfite restriction analysis (COBRA) using methylation-specific restriction enzymes, or the MethylDetector kit (Active Motif), are less than optimal for single blastocyst analyses, resulting in insufficient recovery of DNA, PCR bias and lack of reproducibility. An alternative approach to single oocyte and early embryo methylation analysis utilized the EZ-DNA Methylation kit (Zymo Research) with a limited dilution bisulfite pyrosequencing technique[14], although the reported success rates of bisulfite converted DNA amplification was lower than the method described here.

Bisulfite mutagenesis is the gold standard for analyzing DNA methylation, and it is clear that the analysis of single cells is an important step forward. Agarose embedding permits smaller sample sizes to be analyzed with bisulfite treatment, as agarose protects against DNA degradation and prevents DNA loss during numerous protocol steps. However, when compared to the blastocyst, a single oocyte has approximately 3-6 pg of genomic DNA. Our modified protocol, which embeds single oocytes in agarose containing lysis buffer, prevents DNA loss beginning at the initial lysis step and moderates the harsh bisulfite treatment. In summary, the advantage of single oocyte analysis is that it allows detection of inadvertent cumulus cell contamination as well as unmasking rare events and eliminating any PCR biases in pooled samples. Altogether, this modified protocol provides quality data on the methylation state of individual oocytes and early embryos.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by the University of Western Ontario, the Department of Obstetrics and Gynaecology; and a grant ER06-02-188 from the Ministryof Research and Innovation, Early Researcher Award. MMD was supported by a CIHR Training Program in Reproduction, Early Development and the Impact on Health (REDIH) Graduate Scholarship.

References

1. Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33 Suppl.**, 245-254 (2003).
2. Rodenhiser, D. & Mann, M. Epigenetics and human disease: translating basic biology into clinical applications. *CMAJ.* **174**, 341-348 (2006).

3. Frommer, M., *et al.* A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1827-1831 (1992).
4. Clark, S.J., Harrison, J., Paul, C.L., & Frommer, M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* **22**, 2990-2997 (1994).
5. Feil, R., Charlton, J., Bird, A.P., Walter, J., & Reik, W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. *Nucleic Acids Res.* **22**, 695-696 (1994).
6. Raizis, A.M., Schmitt, F., & Jost, J.P. A bisulfite method of 5-methylcytosine mapping that minimizes template degradation. *Anal. Biochem.* **226**, 161-166 (1995).
7. Patterson, K., Molloy, L., Qu, W., & Clark, S. DNA Methylation: Bisulphite Modification and Analysis. *J. Vis. Exp.* (56), e3170, DOI: 10.3791/3170 (2011).
8. Olek, A., Oswald, J., & Walter, J. A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res.* **24**, 5064-5066 (1996).
9. Mann, M.R., *et al.* Selective loss of imprinting in the placenta following preimplantation development in culture. *Development.* **131**, 3727-3735, (2004).
10. Market-Velker, B.A., Zhang, L., Magri, L.S., Bonvissuto, A.C., & Mann, M.R. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum. Mol. Genet.* **19**, 36-51 (2010).
11. Meng, L.H., Xiao, S.Q., Huang, X.F., Zhou, Y., & Xu, B.S. [A study on bisulfite sequencing method for methylation status of imprinted genes in single human oocytes]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi.* **25**, 289-292 (2008).
12. Denomme, M.M., Zhang, L., & Mann, M.R. Embryonic imprinting perturbations do not originate from superovulation-induced defects in DNA methylation acquisition. *Fertil. Steril.* **96**, 734-738 (2011).
13. Tahiliani, M., *et al.* Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science.* **324**, 930-935 (2009).
14. El Hajj, N., *et al.* Limiting dilution bisulfite (pyro)sequencing reveals parent-specific methylation patterns in single early mouse embryos and bovine oocytes. *Epigenetics.* **6**, 1176-1188 (2011).

