# PROTOCOL FOR:

## Simple workflow for genome and methylation analyses of ejaculated bovine spermatozoa with low sperm input.

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## LEGEND

***ATTENTION***

\* ***HINT***

***REST***

# REAGENTS AND MATERIALS

NaCL

EDTA

TRIS-CL

SDS

DDT

Proteinase K

Percoll

GoTaq Hot Start Green Master mix (2x) (Promega, Madison, WI, USA)

EpiTect Bisulfite Kit (#5904)

QIAquick PCR purification kit (Cat. #28104; Quiagen, Hilden, Germany)

HEPES Buffered Saline (10X and 1X)

# PROCEDURE

SPERM SEPARATION

1. Prepare two 45:90 Percoll gradients according to the following: (instructions for a single gradient)
* Prepare a 90% Percoll solution:
	1. Place 1.35 mL of Percoll in a 10mL conical tube
	2. Add 0.25 mL of 10X HBS or PBS
* Prepare a 45% Percoll solution:
	1. In a separate 10mL conical tube, place 0.5mL of 1X HBS or PBS
	2. Remove 0.5mL of 90% Percoll and add it to the new tube containing 0.5mL of 1xHBS/PBS
* Gently layer 1mL of the 45% Percoll on top of the 95% Percoll to generate the 45/90 gradient
* Warm for 15 min at 37°C
1. Thaw two 0.5mL straws of bull sperm in a water bath at 37°C for 30 sec
2. Gently layer a single straw on top of each 45/90 Percoll gradient
3. Centrifuge at 2500 x G for 10 min
4. Remove supernatant down to the sperm pellet and resuspend in 4mL of 1X HBS/PBS
5. Centrifuge at 2500 x G for 5 min and remove supernatant
6. Add 95µL of 1XHBS/PBS to each pellet and resuspend
7. Combine resuspended sperm into a single tube and add 300µL of 1X HBS/PBS
8. Determine concentration of sperm and extend to 4 x 106 total motile sperm in 400µL of HBS or

sperm holding medium such as TALP.

DNA EXTRACTION FROM BULL SPERM

1. **Sperm Lysis**
2. Add 400µL of sperm prep to 3.6mL of Buffer SW (see Recipe’s below)
3. Vortex 10sec full speed
4. Centrifuge 2500 x G for 5 min
5. Remove supernatant to ~1mL
6. Vortex full speed 10 sec
7. Transfer sample to 2.0mL microcentrifuge tube
8. Centrifuge 17,000 x G for 2 min
9. Remove all supernatant without disturbing the sperm pellet
10. Resuspend the pellet in 220µL of Buffer SL (see Recipes below) with the addition of 30µL of DDT (100mM final concentration)
11. Add 50 µL Proteinase K (40mg/ml)
12. Incubate sample for 2hr at 55°C on dry heat
13. **DNA purification**

**Note: All the centrifugation steps are performed at 9000rpm unless stated otherwise. Any silica columns designed for DNA extraction are likely suitable for DNA purification. Columns with limited volume may require multiple passage to accommodate sample size.**

1. Add 500µL Buffer E (optimized)and incubate 10 min at 55°C.
2. Add 400µL of ethanol to the sample lysate, mix by vortexing and transfer to the DNeasy spin columns (Qiagen).
3. Centrifuge the column for 1 min and discard the flow
4. Add 500µL Buffer WB1 and centrifuge 1 min -discard flow
5. Add 500µL Buffer WB2 and centrifuge 1 min -discard flow
6. Add 750 ul of buffer WB2 and centrifuge at 14000 rpm for 3 min. Discard the flow and transfer the column to new collection tube and centrifuge for additional 1 min to completely dry the column.
7. Transfer column to a new 2.0mL microcentrifuge tube
8. Add 23µL of RNAse free H2O and let rest for 1 -5 min at room temperature to increase DNA yield
9. Elute by centrifugation for 1 min
10. Repeat elution to increase yield in a separate tube
11. Determine DNA yield and optical density by NanoDrop or Qubit Assay.

***REST -*** Store samples at -20°C

BISULFITE CONVERSION OF SPERM

From EpiTect Bisulfite Kit (#5904) – Follow manufacture instructions for reagent preparation and storage

* For each sample use a 200µL PCR Tube and add:

🡪 (50ng max used)-Limits not tested

|  |  |
| --- | --- |
| DNA | Up to 20µL |
| H2O | Up to 20µL  |
| Bisulfite Mix | 85µL |
| DNA Protect Buffer | 35µL |
| Total Volume | 140µL |

* Continue conversion in thermocycler according to manufacture specified protocol
* \* ***HINT For final elution: Use 20µL of RNAse free H2O***
* Repeat elution in new tube and determine DNA yield by NanoDrop RNA (Factor 4) settings

PCR AMPLIFICATION

\* ***HINT****: PCR conditions and primer concentrations may vary with final amplicon size*

1. Prepare 20 µL reaction volumes consisting of 10 µL of GoTaq Hot Start Green Master Mix, 50-100ng of DNA template, 0.4 µL (4 pM) of reverse and forward primer and H2O) up to 20µL
2. PCR conditions: 95° for 5 min | 95° for 30 sec | 59° for 30 sec | 72° for 45 sec | 35 cycles | 72° for 7 min
3. Prepare a 2nd round of PCR consisting of 5 µL of 1st round PCR product, 4.2 µL nuclease free H20, 10 µL of master mix, 0.4 µL of forward and reverse primer
4. Use identical PCR conditions as previous for second round

GEL IMAGING

1. Load 5 µL of final PCR product onto a 0.9% agarose gel with EtBR (0.5µg/mL) and 0.5X TBE buffer
2. Add ladder appropriate for expected amplicon size
3. Run gel at 95V for 60 min and image gel

***ATTENTION:*** *Additional run times may be necessary to confirm the amplification of a single, or multiple bands.*

PCR PURIFICATION

\* ***HINT****: PCR purification should be used in place of gel extraction when the successful amplification of a single band is present.*

1. Remaining PCR product can be purified using a QIAquick PCR Purification Kit following manufacture specifications with the below modification:

***ATTENTION:*** *For final elution, add 15 µL of nuclease free water to the center of elution tubes. The addition of larger volumes of water may result in a sample too dilute for downstream sequencing. Repeat elution for higher yields.*

SEQUENCE PREPARATION

1. Quantify DNA using a NanoDrop and observe purity
2. Submit a minimum of 10-100ng of DNA with purity of 1.8-1.9 based on a 260/380 nM absorbance ratio

\* ***HINT****: Final primer concentration, sample volume and DNA template may be specific to the sequencing facility.*

# RECIPES

10X HBS (100ml)

NaCL 7.95g

KCL (1M) 4ml

CaCl2 (1M) 1mL

MgCl2 (1M) 5mL

Fructose 2.523g

Fatty Acid-free BSA 5.0g

Buffer SW (100mL)

NaCL (150mM) 0.876g

EDTA (10mM) 2.0mL

Buffer SL

100 mM TRIS-CL, 10 mM EDTA, 500 mM NaCL, 1% SDS

Buffer E

5M Guanidium Thiocyanate, 50mM Tris-HCL(pH 8.0), 25mM NaCl

WB1

3M Guanidium Thiocyanate, 100mM Tris-HCL(pH 8.0,) 20mM EDTA, 50% ethanol

WB2

10mM Tris-HCL (pH 7.4), 1mM EDTA, 100mM NaCl, 80% ethanol

PCR Reaction 1 (20 µL)

Reagent Volume Final concentration

GoTaq Hot Start Master Mix (2) 10.0 µL 1 x

DNA Template 9.2 µL 50 – 100ng

Primer (forward) 0.4 µL 4 pM

Primer (reverse) 0.4 µL 4 pM

Combine reaction in a PCR tube, mix gently by pipette and centrifuge ~ 3 sec

PCR Reaction 2 (20 µL)

GoTaq Hot Start Master Mix (2) 10.0 µL 1 x

DNA Template 5.0 µL

Primer (forward) 0.4 µL 4 pM

Primer (reverse) 0.4 µL 4 pM

Nuclease free H2O 4.2 µL

Combine reaction in a PCR tube, mix gently by pipette and centrifuge ~ 3 sec

# EQUIPMENT

NanoDrop (ThermoFisher Scientific, Waltham, MA, USA)

# TROUBLESHOOTING

For samples that yield low or no detectable DNA after elution, perform PCR regardless and check for amplification of desired DNA locus.