Experimental Protocol – DNA isolation and WGS of nasal swabs, blood and/or tissues from sheep

Summary

This experimental protocol describes the generation of WGS libraries from nasal swabs, blood and/or frozen sheep tissues at the Roslin Institute. The protocol was developed to assemble *de novo* genome assemblies and to supplement the investigation of gene expression and regulation in Texel x Scottish Blackface sheep at different developmental time points in relation to growth traits. The experiment was split into six steps that are described below.

STEP 1

Taking Nasal Swabs from Large Animals for DNA Isolation

1 Purpose / Introduction

To collect nasal swabs from large animals for downstream isolation of DNA for the purpose of genotyping or other analyses.

2 Equipment/Reagents/Materials

Nitrile Gloves

Performagene Nasal Swabs (PG-100) DNA Genotek

3 Principle

Nasal swabbing is a non-invasive procedure for collection of DNA from animals. It requires minimal restraint of the animal, normally sheep, for a period of a maximum of one minute while the sample is being collected. The nasal swabs can be used to isolate DNA for genotyping to assess parentage, SNP analysis or other DNA analyses.

4 Procedure

1. The animal should be gently restrained by an animal technician at the large animal unit.
2. Remove the swab from the plastic wrapping taking care not to touch anything.
3. Holding the tube, rub the nasal swab inside the animals nostril for up to 5 seconds. Ensure the sponge looks wet and is coated with the nasal sample to achieve the best DNA collection.
4. Hold the tube upright and unscrew the cap from the tube.
5. Turn the cap upside down and place the nasal swab in the tube and screw the cap on tightly.
6. Invert and shake the tube vigorously 10 times to thoroughly mix the sample.
7. Clearly write the animal's ear tag number on the tube, the species and the date of collection.
8. The swabs can be stored at room temperature for up to one year. If samples are stored for periods > one year, they should be stored at -20°C.

5 Risk assessment

1. Care should be taken when working around live animals as they have the potential to bite and kick. A skilled farm animal technician should always be present to restrain the animal when nasal swabs are being taken.
2. Suitable PPE should be worn including nitrile gloves, lab coat or boiler suit and appropriate footwear for working around large animals.

STEP 2

Collection of blood and/or tissue for WGS from large animals

1 Purpose / Introduction

To collect blood samples from large animals for subsequent extraction of DNA for sequencing.

2 Equipment/Reagents/Materials

50ml and 10ml Syringes

18G needles

BD Vacutainer® Blood Collection Tube containing EDTA (purple top)

1.5ml screw top Eppendorf tubes

Nitrile Gloves

15ml Falcon tubes containing 5ml RNALater solution

Cryotubes for snap freezing tissues

Liquid Nitrogen dewars filled with liquid nitrogen

No.11 Swan Morten Scalpels

No. 22 Swan Morten Scalpels

Sterile Plastic Forceps

Sterile Scissors

Sterile PBS in Litre bottles and 50ml falcon tubes

Dry Ice

Wet Ice

Ziplock bags

Chemical Waste Bags

Sterile Funnel

50ml Falcon Tubes

Petri Dishes

Cable ties

Sharps Bins

3 Principle

Blood samples are routinely used to provide material for sequencing animals. From live animals a small sample of venous blood can be collected by a veterinarian. For post mortem collection of tissues, a veterinarian and at least two farm technicians carry out each post mortem. Tissues are handed to those on the tissue collection team to cut into small pieces (<0.5cm diameter). For

preservation of RNA the chopped up tissues are either snap frozen in liquid nitrogen or transferred to a 15ml falcon tube containing 5ml of RNALater solution.

4 Procedure for the collection of blood

1. A veterinarian and at least two farm technicians, to restrain the animal, will be present for this procedure.

2. The veterinarian will collect the venous blood using the needle and syringe into a BD Vacutainer containing EDTA. Ideally 5mls would be collected into one tube and shaken gently. Care should be taken to ensure this is within the home office licence maximum allowable volume (up to 10% of the circulating blood volume) if the animal is under licence.

3. The animal's ear tag number on the tube, the species and the date of collection should be clearly written on the tube.

4. The collected sample is then returned to the lab, aliquoted into 1.5ml screw top Eppendorf tubes and stored at -80°C for future analysis.

5 Procedure for the collection of large animal tissues

1. All surfaces should be cleaned with disinfectant then RNAlater and all participants should wear gloves and ensure they regularly change these gloves throughout the harvest.

2. Tissues should arrive from the post mortem team on a plastic petri dish. The tissue should be cleaned of any excess blood or gut contents (for GI tract tissues only) by dipping in sterile PBS using plastic forceps. A No. 22 Swan Morton disposable scalpel should then be used to chop the tissue into small pieces that are <0.5cm diameter in any direction. To secure the tissue for chopping either a No. 11 Swan Mortem Scalpel or sterile plastic forceps can be used. A new set of scalpels should be used for each tissue. For lipid rich tissues such as brain and adipose pieces should be placed singly and evenly in cryovials, which are immediately snap frozen in liquid nitrogen. All other tissues after being chopped finely are placed in a 15ml falcon tube containing 5ml of RNALater solution. At the end of the post mortem the snap frozen tissues are transferred carefully onto dry ice using a slotted spoon for transport up to the Roslin Institute and filing into the -155°C freezer. The RNALater tubes are transported to Roslin at room temperature then placed in the 4°C cold room for a minimum of 12 hours and a maximum of one month.

3. In addition, some members of the tissue collection team may be involved in dissection of the animal. These individuals will use scissors and scalpels to dissect out the desired tissues.

STEP 3

Purification of DNA from nasal swab samples using the Performagene PG-AC4 Kit

1 Purpose / Introduction

To purify DNA using the Performagene PG-AC4 kit from nasal swab samples collected from animals using PG-100 nasal swabs.

2 Equipment/Reagents/Materials

PG-AC4 reagent package: PG-L2P purifier

Microcentrifuge capable of running at 15,000 × *g*

Air or water incubator at 50°C (Note: The false bottom tube will float in a water incubator, therefore an air incubator may be preferred.)

Ethanol (95% to 100%) at room temperature

DNA buffer: TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0)

(Optional) Glycogen (20 mg/mL) (e.g., Invitrogen Cat. No. 10814-010)

Ethanol (70%) at room temperature

5M NaCl solution

3 Principle

To purify DNA from a 0.5 mL aliquot of a sample that has been collected and preserved in Performagene chemistry with the PG-100 collection kit. The sample is first heated to release the DNA and inactivate inhibitors. A purifier is then added to precipitate impurities and inhibitors, purifier is then added to precipitate impurities and inhibitors, which are removed by pelleting leaving the DNA in suspension. The DNA is then collected by precipitation using NaCl and washed to remove any residual impurities and then used down stream for genotyping or other DNA analysis.

4 Procedure

1. Mix the sample by shaking vigorously for 5 seconds.
2. Incubate the sample in a 50°C air incubator for a minimum of 2 hours, or in a 50°C water incubator for a minimum of 1 hour.
3. Removal of collection sponge: Remove the cap and press the collection sponge against the inside of the tube to extract as much of the sample as possible. Discard sponge and cap.

4. Transfer 500 µL of the mixed Performagene sample to a 1.5 mL microcentrifuge tube. (TRANSFER THE REMAINDER OF THE SAMPLE TO A NEW 1.5ml MICROCENTRIFUGE TUBE AND STORE AT -20**°**C)
5. Add 20 µL (1/25th volume) of PG-L2P purifier to the microcentrifuge tube and mix by vortexing for a few seconds.
6. Incubate sample on ice for 10 minutes.
7. Centrifuge at room temperature for 5 minutes at 15,000 × *g*.
8. Carefully transfer the clear supernatant with a pipette tip into a fresh microcentrifuge tube. Discard pellet.
9. To 500 µL of supernatant, add 25 µL (1/20th volume) of 5M NaCl, mix.
10. To 500 µL of supernatant, add 600 µL of room temperature 95% to 100% ethanol. Mix gently by inversion 10 times.
11. (Optional) Add Glycogen at 20mg/ml as a carrier to increase visibility of the pellet.
12. Allow the sample to stand at room temperature for 10 minutes to allow the DNA to fully precipitate.
13. Place the tube in the centrifuge in a known orientation (DNA pellet may not be visible after centrifugation). Centrifuge at room temperature for 2 minutes at >15,000 × *g*.
14. Carefully remove the supernatant with a pipette tip and discard it. Take care to avoid disturbing the DNA pellet.
15. Carefully wash the DNA by adding 250 µLof 70% ethanol. Let stand for 1 minute at room temperature. Remove the ethanol with a pipette tip without disturbing the pellet.
16. Centrifuge for 6 seconds to pool any remaining ethanol, remove with a pipette tip.
17. Add 100 µL of DNA buffer (e.g. TE buffer) to dissolve the DNA pellet. Vortex for at least 5 seconds. Let sit at room temperature overnight to ensure complete rehydration of the DNA.
18. DNA can now be quantified and used in downstream applications or stored at -20°C.

5 Maintenance

The temperature of incubators and freezers should be checked on a regular basis to ensure they are in correct working order.

Buffers and solutions should be kept fresh and discarded when they become old.

6 Health and Safety

PG-L2P Purifier contains Potassium Chloride refer to the MSDS.

STEP 4

Extraction of genomic DNA from frozen tissue samples

1 Purpose / Introduction

To purify DNA using the Qiagen DNeasy Blood & Tissue Kit from either blood or frozen tissue samples.

2 Equipment/Reagents/Materials

Qiagen DNeasy Blood & Tissue Kit

Ethanol (95% to 100%) at room temperature

1.5ml Eppendorf tubes

Microcentrifuge capable of running at 15,000 × *g*

Waste containers

3 Principle

To purify DNA from either a 0.1mL blood aliquot or a frozen tissue sample for sequencing or long term storage.

4 Procedure

1. For the isolation of DNA from tissue samples, the 'Purification of Total DNA from Animal Tissues (Spin-Column Protocol)' protocol was followed according to manufacturer instructions stated in the 2020 Qiagen DNeasy® Blood & Tissue Handbook.

2. Place the DNA on ice and measure the DNA concentration and purity using the NanoDrop™ 8000 Spectrophotometer and/or Qubit® dsDNA BR Assay Kit according to manufacturer's protocols. Store remaining DNA stock either at 4°C for short term storage or -20°C for longer term storage.

STEP 5

Extraction of genomic DNA from whole blood

1 Purpose / Introduction

1.1 This procedure is intended to purify high molecular weight DNA from 5-10mL of fresh or frozen whole blood, and was originally written by Laura-Lee Boodram.

2 Equipment/Reagents/Materials

Refrigerated benchtop centrifuge, RT microfuge

Shaking water bath at 55°C

Vortex mixer

Buffer A Red blood cell lysis buffer

final concs	**1 litre**	**500ml**
0.32 M sucrose	109.536g	54.768g
10 mM Tris HCl	10mls of 1M	5mls of 1M
5 mM $MgCl_2$ ($6H_2O$)	1.017g	0.508g

Adjust pH to 7.6

Autoclave or Filter sterilise 0.22u prior to addition of Triton-X-100

0.75% Triton-X-100	7.5mls	3.75mls

Buffer B Proteinase K buffer

final concs	**1 litre**	**500mls**
20 mM Tris-HCl	20mls of 1M	10mls of 1M
4 mM Na_2EDTA	8mls of 0.5M	4mls of 0.5M
100 mM NaCl	20mls of 5M	10mls of 5M

Adjust pH to 7.4 autoclave or Filter sterilise 0.22u

10% SDS

Proteinase K 20mg/ml (defrost -20°C stock solution) read COSHH MSDS

Sterile water (chilled overnight)

Isopropanol (-20°C) read COSHH MSDS

70% Ethanol read COSHH MSDS

Haz-Tabs prepare 2.5% bleach solution read COSHH MSDS

3 Principle

3.1 To extract high molecular weight DNA from fresh or frozen blood, red cells are lysed and white cells are digested overnight in buffer with SDS and proteinase K. Salt is then added to precipitate digested protein and cell debris, leaving DNA in solution. DNA is precipitated with isopropanol then washed in 70% ethanol to remove salt and contaminants. The DNA is dried to remove ethanol and re-suspended as required for downstream use.

4 Procedure

1. Add 1 volume of buffer A to 1 volume of blood and 2 volumes of cold, sterile, distilled, deionised water in a sterile, conical, 50ml tube. Vortex gently or invert tube 6-8 times and leave to incubate on ice for 2-3 minutes.

2. Spin at 3500 rpm for 15 minutes at 4ºC. Discard supernatant into 2.5% bleach solution and re-suspend pellet (vortex for 30 seconds at medium speed) in 2 ml of buffer A and 6 ml of water. Spin at 3500 rpm for 15 minutes at 4ºC. The pellet should be white to cream in colour. If pellet is significantly red, repeat washing step again.

3. Add 5 ml of Buffer B and 500 µl of 10% SDS to pellet. Re-suspend pellet by vortexing vigorously for 30 seconds. Then add 50 µl of Proteinase K solution (20mg/ml).

4. Leave to incubate for at least 2hrs or up to overnight at 55ºC in a shaking water bath. Remove samples and leave to cool to room temperature (or leave for 2-3 minutes on ice). Add 4 ml of 5.3M NaCl solution. Vortex gently for 15 seconds.

5. Spin at 4000 rpm for 20 minutes at 4ºC. Pour off supernatant containing DNA into a fresh 50 ml tube. Take care not to dislodge pellet. Add an equal volume of cold isopropanol (stored at

-20ºC). Invert gently 5-6 times to precipitate DNA.

6. Remove DNA with a wide bore tip and transfer to 5 ml 70% EtOH in a 15ml tube. Wash pellet by inverting several times.

7. Transfer pellet with a wide bore tip to 1 ml 70% EtOH in a microfuge tube and wash by inverting tube.

8. Spin at 10,000g 5mins in RT microfuge. Remove EtOH and leave DNA to air dry for 15-20 minutes at RT.

9. Re-suspend in 300-400 µl of Tris-HCl or TE pH 8.0 as required. Leave to re-dissolve for a few hours at RT. Transfer to 4ºC. Mix by inversion before quantitation.

5 References

Protocol online, Extraction of genomic DNA from whole blood, author Laura-Lee Boodram 2004.

Helms, C. Salting out Procedure for Human DNA extraction. In The Donis-Keller Lab - Lab Manual Homepage [online]. 24 April 1990. [cited 19 November 2002; 11:09 EST]. Available from: http://hdklab.wustl.edu/lab_manual/dna/dna2.html.

Epplen, J.E., and T. Lubjuhn. 1999. DNA profiling and DNA fingerprinting. Birhkhauser Verlag, Berlin. p.55.

STEP 6

WGS library preparation and sequencing

Genomic DNA samples that passed QC were sent to Novogene, Cambridge UK, who prepared NEB Next Ultra DNA Library Prep Kit for Illumina (E7370) DNA libraries for sequencing on the Illumina NovaSeq 6000 according to the standard work flow:

https://www.neb.com/protocols/2014/05/22/protocol-for-use-with-nebnext-ultra-dna-library-prep-kit-for-illumina-e7370