Preparing agarose plugs from blood samples.

- Blood should not be older than 5 days.
- Take 1 mL of bloodplasma (only from Heparine tubes), spin for 1 minute full speed and bring supernatant in a clean tube and store at -80°C
- Fresh blood: Invert the EDTA blood tube several times to get a homogeneous solution (For frozen blood: First defrost at 37°C)
- Transfer the blood (10 to 20mL) to a labelled 50 mL tube, rinse the blood tube with 1x erythrocyte lysis buffer, and add this to the blood to a final volume of 50 mL. Mix gently.
- Put the tube on ice for 5-15 minutes until the red blood cells are lysed (depending on vitality of the cells (only 5 minutes if cells have been frozen).
- Spin the lymphocytes down for 8 minutes at 1600 rpm in a Beckman centrifuge (type GS-6R; 15°C, brake high).
- **Warning 1; don't leave the cells pelleted, go quickly to the next step**
- Discard the supernatant (leave 5 mL).
- **Warning 2; If white blood cell pellet not visible, do not discard supernatant but homogenize this again and repeat lysis and centrifuge steps**
- Resuspend white blood cell pellet in 5 mL supernatant by scratching bottom tube over irregular surface.
- Add erythrocyte lysis buffer to a volume of 30 mL and after homogenising bring 15mL into a 15mL tube (for plug DNA), leaving 15 mL in 50mL tube (liquid DNA)
- Put the tube on ice for 5 minutes.
- Spin the lymphocytes down for 8 minutes at 1600 rpm in a Beckman centrifuge
- **Warning 3; don't leave the cells pelleted, go quickly to the next step**
- **Preparation plug DNA (from 15 mL tube)**
- Melt the SE/1.4% agarose and put the tube at 60°C in a water bath to avoid coagulation of the agarose.
- Put tape on one side of the plastic mold and place mold on ice.
- Add the calculated volume of SE (about 1 to 1.5mL for 10 mL of blood) to the pellet from the **15 mL tube** and resuspend with a blue tip until the pellet is completely dissolved
- Add an equal volume of SE/1.4% agarose and resuspend gently.
- Dispense mixture over slots plastic mold with a blue tip and leave the plugs to coagulate.

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Calculation amounts of SE and SE/1.4% agarose:

10 mL of blood: $14\text{-}28*10^6$ cells = 100 to 200 μg DNA
Approximately 7 μg DNA per plug (half plug per digestion) = approx $1*10^6$ cells
Accurate determination of the number of white blood cells can be done by using the size of the white blood cell pellet
Volume 1 plug is 120 μl: 60 μl resuspended cells and 60 μl SE/1.4% agarose

Example for 10 mL of blood:
10 mL of blood: $20*10^6$ cells → 20 plugs
20 x 60 μl = add 1200 μl SE and resuspend pellet
add 1100 μl SE/1.4% agarose

- Prepare a solution of 10 mL SE/sarcosyl (1%) with 300 μl pronase in a 15mL tube.
- Remove the surplus of agarose, when coagulated, from the mold and push the plugs out of the plastic mold into the SE/sarcosyl/pronase solution with a pipetting balloon for Pasteur pipettes (air pressure).
- Incubate the plugs in a 37°C water bath for at least two days.
  At day three, discard the SE/sarcosyl/pronase and replace with 10mL H_2O.
- Discard the H_2O add 10 mL 0.5M EDTA to the plugs.
- Store the plug DNA at 4° C.

- **Preparation liquid DNA (from 50 mL tube)**
  - Meanwhile (when cells for plug DNA are in mold) resuspend the other pellet (**50mL tube**) with 3mL nucleus lysis buffer and add 150 µL 20 % SDS and 100 µL pronase and leave it for at least 2 days at 37°C
  - After incubation add 1.2mL 5M NaCl to the lysed cells, shake violent
  - Divide over two 2 mL eppendorf tubes and spin the precipitated proteins down for 15 minutes at full speed (15 K)
  - Bring supernatant to clean 15 mL tube without the proteins.
  - Add 10mL 100% EtOH, shake and fish the precipitated DNA.
  - Wash DNA in 70% EtOH solution
  - Dissolve the DNA in 250µL TE^{-4} (for 10mL of blood).
  - 15 minutes 65°C (Dnase inactivation and dissolving)
  - Store liquid DNA at 4° C.

- 20x Erythrocyte lysis buffer (2 L):
  331.6 g NH_4Cl
  40.0 g $KHCO_3$
  80 mL 0.5 M EDTA

  1x Erythrocyte lysis buffer:
  155 mM NH_4Cl
  10 mM $KHCO_3$
  1 mM EDTA pH 8.0

- 1x Nucleus lysis buffer (1 L):
  33 mL TRIS-HCl (pH 8.2)
  4 mL 0.5 M EDTA
  80 mL 5 M NaCl

  1x Nucleus lysis buffer:
  10 mM TRIS.HCl (pH 8.2)
  2 mM EDTA
  400 mM NaCl

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- SE:
  500 mL 7.5 mL 5 M NaCl
  25 mL 0.5 M EDTA pH 8.0
  467.5 mL H_2O

- SE/1.4% agarose:
  Dissolve 0.14 g InCert agarose in 10 mL SE
  InCert agarose (1 g FMC BioProducts, catalog no. 50121, www.bioproducts.com)

- 10x Stock SE/10% sarcosyl (dilute in water)
  100 mL 15 mL 5 M NaCl
  50 mL 0.5 M EDTA pH 8.0
  10 g sarcosyl (N-lauroyl-sarcosine)
- **Warning; use hood to weight sarcosyl**
  add mL H_2O to 100 mL

- Pronase (20 mg/mL):
  Dissolve pronase in 10 mM NaCl/10 mM Tris-HCl (pH7.5) to a final concentration of 20 mg/mL. Incubate 1 h at 37°C. Store at –20°C.

- TE^{-4}:
  10 mM Tris-HCl pH 7.4
  0.1 mM EDTA pH 8.0

  1 litre:
  5 mL 2 M Tris-HCl pH 7.4
  0.2mL 0.5 M EDTA pH 8.0

- EDTA:
  Add 186.1 g of disodium ethylenediaminetetra-acetate.2 H_2O to 800 mL of H_2O. Stir vigorously on a magnetic stirrer. Adjust pH to 8.0 with NaOH.
  Note: the disodium salt will not go into solution until the pH is adjusted to approximately 8.0 by addition of NaOH.