SEMEN COLLECTION, EVALUATION & FREEZING PROCEDURES

For: Cheetah Conservation Fund; December 2017.

PREPARATION supplies:

• On slide warmer:
  1. Prepared medium (*see recipe)
  2. Slides and Coverslips
  3. Sterile 3mL tubes; Sterile 1.5mL Eppendorf tubes
  4. Sterile collection cups
  5. Black Styrofoam rack for holding Eppendorf tubes

• Collection area:
  1. Electroejaculation (EE) box
  2. Clean 1.6 & 1.9 probes (sand paper if necessary)
  3. KY Jelly
  4. Calipers, Gauze & Gloves
  5. EE data sheet & Pen

• Microscope area:
  1. pH indicator paper
  2. Semen evaluation form on clipboards (pens & sharpies)
  3. Sterile blue & yellow micropipette tips
  4. Sperm pipettes (20, 200 & 1000 μl)
  5. Unopette
  6. Hemocytometer
  7. Tally counter
  8. Calculator

• Freezing Preparation:
  1. Straws & Labels
  2. Canes & Labels
  3. Goblets
  4. Sleeves
  5. Cryo-pens or permanent marker
  6. Test-Tube rack
  7. Working tank of liquid nitrogen
  8. Sperm storage liquid nitrogen tank
  9. Refrigerator or Styrofoam cooler with Ice packs
  10. Heat Sealer

PREPARATION reagents:

• TEST:
  1. Both 0% and 12% TEST are in the vet clinic freezer (in WHITE cardboard box). TEST is very expensive, thus appropriate care should be taken to prevent wasting it.
    ▪ Ideally, wait to thaw TEST until there is confirmation that there will be sperm to freeze. This prevents thawing of TEST that cannot be refrozen and then must be discarded.
    ▪ But remember that: TEST must be at room temperature before use.

• EXTENDER Medium:
  1. See next page for recipe.
    ▪ You can prepare most of the mix prior to the workup – but hold off on opening any new Ham’s bottle (see detail on next page).
EXTENDER MEDIUM recipe:

- 9.2mL Ham’s F10 with Hepes
  - If using Ham’s Basal F10 medium (without Hepes), use 9mL of Ham’s Basal medium + 200μL of Hepes (1mol/L) solution.
- 100μl glutamine
- 100μl pyruvate
- 100μl penicillin/streptomycin/neomycin (P/S/N) antibiotic mix
- 500μl fetal calf/bovine serum (FCS or FBS)

10mL total

-Combine above ingredients into a 12mL sterile snap cap tube and mix gently.
-Connect a 10mL syringe to a 0.22μm filter (ideally luer lock). Carefully pour the medium into the top of the syringe and then insert the plunger. Filter the medium into another 12mL sterile snap cap tube.
-Keep warm on the slide warmer until needed.

NOTES:
-Ham’s F10 (with Hepes):
  - Do not open a new Ham’s unless you are certain that you will use it (it only stays good for 6-8 weeks after the bottle is opened).
  - Ham’s is stored in the clinic refrigerator
  - Mark the date on which the bottle was opened (as for any reagent)
  - If Ham’s scheduled to be used within 5 weeks, simply parafilm the top of the bottle. If not all will be needed, store part of the remaining Ham’s medium (e.g. 30ml) in labelled 10mL syringes to prolong life span. Ensure no air in syringe + place syringe stop on open end and parafilm. Life expectancy >7 weeks.
  - Discard medium after the bottle has been open for 8 weeks – or when there is a noticeable color change (pink→cloudy yellow-orange)

-Hepes:
  - Stored in the clinic refrigerator in small vial with orange screw cap

-Glutamine, Pyruvate and P/S/N Antibiotic:
  - Pre-made aliquots are stored in the -20°C clinic freezer.
  - For each batch of working medium, thaw one aliquot of glutamine, pyruvate and antibiotic
  - Aliquots are ~125μL

-Fetal Calf/Bovine Serum (heat inactivated):
  - Pre-made aliquots are stored in the -20°C clinic freezer.
  - For each batch of working medium, thaw one aliquot of FCS
  - Aliquots are ~550μL
  - There are also larger aliquots of FCS in both clinic freezer and the walk-in freezer inside the meat room
  - If a larger tube is thawed, smaller aliquots need to be prepared and refrozen.

-If you need to let a sperm sample sit for longer than the protocol suggests (to process multiple animals or due to unforeseen schedule conflicts), this is not ideal, but if needed it would be better to pause after the addition of TEST, once samples are placed in the refrigerator.
I. SEMEN COLLECTION

*These procedures may be used for cheetahs, African leopards, clouded leopards and other cats of a comparable size.

1. Once the animal is on the table and determined to be in a stable plane of anesthesia, obtain rectal temperature, heart rate and respiration rate.
2. Measure the length & width of both testes with calipers and assess firmness. Record size and firmness of testes on the Electroejaculation (EEJ) Worksheet.
   - Testes Firmness: (1) = hard, (2) = normal, (3) = flaccid
3. Generously lubricate the probe with sperm-friendly lubricant (e.g. KY jelly); don’t use fingers to spread.
   - 1.6 cm probe for smaller animals; 1.9 cm for larger animals
4. Make sure box is turned OFF and the dial is at 0. Insert probe gently into rectum with steady forward pressure. Gently message the sphincter muscles from the outside, which helps with insertion.
5. Position the electrodes such that they are directly over the underlying accessory sex glands (prostate). Avoid inserting it too far as that may induce urination. The central electrode on the probe should be positioned ventrally.
6. Prolapse penis, clean with water and dry well with gauze. Record appearance of penis spines on EEJ Worksheet.
   - Penis Spines: (1) = none, (2) = small/slight, (3) = prominent
   - DO NOT USE KY JELLY ON PENIS!
7. Place warmed sterile collection vial over the penis.
   - Have people inside clinic keeping vials warm with their hands.
8. Determine that the animal is stable and that the health care staff is ready for the stimulations to begin. Signal the EE box operator to begin the procedure.

EEJ generally consists of 2-3 ‘series’. Within a series, there are usually about 30 ‘stimuli’. Each collection cup sent for analysis contains a ‘fraction’ of that day’s collection.

SERIES 1 EXAMPLE:
EEJ is adapted to the reaction of each cat. Series 1 generally consists of 10 stimulations at 2 volts, 10 at 3 volts, and 10 at 4 volts. Any series can be modified to account for size, age, level of anesthesia, semen output, and other factors. Voltage can be stepped down, pending quality of semen collected. For an adult (>2 year) cheetah, maximum recommended voltage is 5 volts and maximum recommended total number of stimulations is 90.

Example of stimuli during series 1:
   - Increase from 0-to-2 volts over a 2-second period ("going up")
   - Hold at 2 volts for 2 seconds ("holding...")
   - Return abruptly back to 0 volts ("going down")
   - REPEAT 10 TIMES
     - Increase from 0-to-3 volts over a 2-second period ("going up")
     - Hold at 3 volts for 2 seconds ("holding...")
     - Return abruptly to 0 volts ("going down")
     - REPEAT 10 TIMES

SERIES 2 and 3 EXAMPLE:
Series 2 & 3 generally consist of 20 stimulations at 3 volts and 10 at 4 volts, but not limited to.

*Rest the animal between each series while processing the semen sample.
*Number of fractions within the series is dependent on the cat as well as the person performing the EEJ.
*Each fraction usually has a minimum of 5 stimuli, otherwise in multiples of 5.
9. While stimulating the animal, apply gentle pressure ventrally to the probe.
10. Between individual stimulations, there should be a 2-second hold at 0 volts.
   ▪ If the probe needs to be repositioned, do so between stimulations and NOT during!
11. Rest the animal between each series while processing the semen sample.
12. Collection cups are usually changed roughly every 5 stimuli. The collector might want
to switch to a clean collection cup in the middle of a series if there is a large volume of
semen in cup.
   ▪ This becomes especially important with animals that are known to urinate during
the procedure. STOP STIMULATIONS WHILE CHANGING CUPS!
13. Good communication between the collector, the box operator and the health care staff
is crucial.
II. SEMEN EVALUATION

From EVERY fraction of each series:

1. Place 3μl of raw semen on a pre-warmed slide and place it on the microscope stage
2. Immediately determine percent motility (visual, subjective assessment) and status of progression, rated 1-5. Record on EE Worksheet.

<table>
<thead>
<tr>
<th>STATUS OF PROGRESSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 T. twitching with no forward progression</td>
</tr>
<tr>
<td>2 Side to side movement with little forward progression</td>
</tr>
<tr>
<td>2.5 Moving forward very slowly or in circles</td>
</tr>
<tr>
<td>3 Moving forward at a slow steady pace but with no circles</td>
</tr>
<tr>
<td>3.5 Moving forward at a quick steady pace</td>
</tr>
<tr>
<td>4 Moving forward rapidly</td>
</tr>
<tr>
<td>5 Moving forward very quickly; hyperactive</td>
</tr>
</tbody>
</table>

3. Remove small volume of raw semen (can even be leftovers off previously used micropipette) and place on pH indicator. Record pH level on EE Worksheet
   ▪ Must check pH for every fraction of every series to determine if sample is contaminated by urine.
   ▪ Healthy pH is between 8.3-8.9
   ▪ If <8.3, then sample may be contaminated.
4. Measure volume of raw semen and transfer to new sterile container (either 3ml tube or 1.5 Eppendorf tube, depending on ejaculate volume) and record amount on EE Worksheet.
5. Extend semen 1:1 with an equal volume of prepared extender medium.
   ▪ Add medium slowly (drop-wise) to semen and mix by gently flicking tube while adding medium.
6. Label and store tube in a black Styrofoam rack so that it is protected from temperature changes and light. (Labeling Example: 1A = series 1, fraction 1; 2C = series 2, fraction 3; etc.)

III. SEMEN ASSESSMENT

1. Keep all labeled extended aliquots separated (in black Styrofoam rack) until the electroejaculation collection is fully completed.
2. IF all samples are comparable they may be combined slowly and gently into a sterile 3ml test tube at this time. Mix well throughout by gently flicking tube.
   ▪ If not comparable, you may want to do a reassessment of motility and status from every fraction of that series.
3. Remove 3μl and load heated slide to observe overall motility and status assessment. Record on EEJ Worksheet.
4. Determine overall sperm concentration:
   a. Remove 10μl of extended sample and place into unopette; mix well
      ▪ If no unopette available, add 10μl of extended sample to 1.990ml water (or 5 μl in to 0.995 μl water); mix very well; let stand at room temperature for 5 minutes.
   b. Let unopette sit at room temperature for 5 minutes until sperm are no longer motile.
   c. Mix well again and load sample into hemocytometer (1 drop on each side) and let sit for another 5 minutes.
   d. Count number of sperm in four corners on each side (8 total corners)
Determination of Sperm Concentration and Test Needed:

Overall motility = 80%
Total raw semen volume (must be in ml) = 550μl = 0.55ml
Total extended (1:1) volume (must be in ml) = 1.1ml
Sperm count on hemocytometer (8 corners) = 24

Raw sperm concentration = (sperm count / 2) x 10^6 = 12 x 10^6 / ml

Total sperm = (raw sperm concentration) * (raw semen volume in mls)
Total sperm = (12 x 10^6) * (0.55) = 6.6 x 10^6

Total motile sperm = (total sperm) * (% overall motility)
Total motile sperm = (6.6 x 10^6) * (0.80) = 5.28 x 10^6

Test volume to add = (total motile sperm) / (60 x 10^6)
Test volume to add = (5.28 x 10^6) / (60 x 10^6) = 0.088 ml or 88μl TOTAL TEST to add to pellet

Test amounts:
If you have a total of 0.088ml (88μl) TEST to add to pellet, then...
...you will need 44μl of 0% TEST and 44μl of 8% TEST
...at this time, you will also need to make the 4% and 8% TEST. Since there is only 0% and 12% TEST to work with, you will have to mix the two to get 4% and 8%. You can achieve this by using a 2:1 dilution of 0%:12% to make 4% or using a 1:2 dilution of 0%:12% to make 8%.

If you need 44μl of the 8% TEST then you can easily make 60μl using the 0% and 12% TEST
- i.e. 60 x (½) = 20μl of 0%
- i.e. 60 x (⅓) = 40μl of 12%

<table>
<thead>
<tr>
<th></th>
<th>0%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>8%</td>
<td>20μl</td>
<td>40μl</td>
</tr>
<tr>
<td>4%</td>
<td>40μl</td>
<td>20μl</td>
</tr>
</tbody>
</table>

*Only prepare 4% TEST if you are using straws with cotton plug seal
*If you have cotton plug sealed straws, you will be using the 4% TEST at a later time, but it is best to make it simultaneously with the 8% TEST to avoid confusion. Label both Eppendorf tubes appropriately.
IV. PROCESSING FOR FREEZING

1. Equally divide total extended semen into sterile Eppendorf tubes to be centrifuged (number of tubes will depend on total volume)
2. Centrifuge tubes for 8 minutes at 1.2 rpm (~100g). While the centrifuge is running, count sperm on the hemocytometer; determine sperm concentration and number of total motile sperm. Record.
3. As soon as centrifuge stops, **immediately** collect the supernatant from each tube and place into separate sterile Eppendorf tubes.
   - Supernatant from each tube may be combined into 1-2 Eppendorf tubes.
   - Remove as much supernatant as possible WITHOUT disturbing the pellet.
4. Re-centrifuge supernatant in Eppendorf tubes for another 8 minutes at 1.2 rpm (~100g).
5. Combine all the pellets into one Eppendorf tube and resuspend pellets by adding the 0% TEST determined by the above equation. Add the room temperature TEST drop-wise, mixing well throughout addition – flicking.
   - In our example you would add 44μl of 0% TEST at room temperature.
6. As soon as the centrifuge stops, again remove the supernatant from each tube, leaving approximately 30-40μl sperm pellet. Discard supernatant and combine new pellets with the sperm already in the 0% TEST. Label the cap of the Eppendorf tube after the cheetah’s name or AJU number.
7. Place the Eppendorf tube containing the sperm sample + 0% TEST as well as the Eppendorf tubes with the 4% and 8% TEST in a room temperature water bath.
8. Place water bath in refrigerator. Monitor temperature approximately every 30 minutes or until the water bath reaches 4-5°C. [Waiting step] At this time you will begin the 8% glycerol TEST addition. *see below.
9. Prepare semen straws with labels (cut if necessary or desired) and place in refrigerator. Label with the cheetah’s AJU number and date. e.g. AJU1444 - 5 June 2013

*8% GLYCEROL TEST ADDITION:*
- Add total volume over a period of 30 minutes in a series of three separate aliquots
- Add each aliquot drop-wise to the semen+0% TEST Eppendorf tube, mixing well throughout.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Equation</th>
<th>Total (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliquot 1</td>
<td>0</td>
<td>44μl x (1/4)</td>
</tr>
<tr>
<td>Aliquot 2</td>
<td>15</td>
<td>44μl x (1/4)</td>
</tr>
<tr>
<td>Aliquot 3</td>
<td>30</td>
<td>44μl x (1/2)</td>
</tr>
</tbody>
</table>
V. FREEZING STRAWS

1. After adding Aliquot 3 of the 8% glycerol TEST to the semen+0% TEST sample, mix it VERY well and check final sperm motility and status by removing 10μl and loading it onto a slide. Record on EEJ Data Sheet.

2. Load sample into prepared straws (~80μl for cut straws or ~125μl for full straws) using a sterile tipped pipette.

3. Pipette ≥5μl of the 4% TEST into the end of the straw that contains the cotton plug and heat seal the end of the straw without a cotton plug.
   - Make sure to leave an air pocket between the TEST-soaked cotton plug and the sperm sample.
   - If using straws without cotton plugs, heat seal both ends of the straw. You would not need to make the 4% TEST in this situation.

4. Take care to keep sample cold (4°C) at all times. Obviously, this will not be possible if using a heat sealer that is not in the refrigerator. In those cases, seal the straws at room temperature as quickly as possible and return them to 4°C.

5. Place the two-tiered metal test-tube rack in the Styrofoam cooler box.

6. Fill the cooler box with liquid nitrogen from the working tank (tank with no samples) up to the predetermined level on the rack. This level will be determined such that there is one tier 3 inches and one tier 1 inch above the liquid nitrogen level.

7. Place the straws evenly on the tier 3 inches above the liquid nitrogen for 1 minute with the Styrofoam cooler box sealed with lid.

8. Using tongs, move the straws quickly to the tier 1 inch above the liquid nitrogen for 1 minute with the cooler box sealed with lid.

9. Using tongs, plunge the straws into the nitrogen, keeping them submerged at all times.

10. Package the straws under the liquid nitrogen into a pre-labeled cane holding the goblet, cover with a sleeve and store into the storage tank inside the meat room freezer. Record on Worksheet and Data Sheet the GRB#, Bucket # and Tank #.
   - Be sure to keep the straws under the liquid nitrogen at all times.

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GENERAL NOTES

Keep very detailed notes on all aspects of the procedure, including anesthesia. Even if you do not think a bit of information is important it could be extremely important to someone else in the future. If in doubt, write it down! Detailed data sheets are crucial and provide continuity in data collection for all samples. Record location of all samples in the nitrogen storage tanks.

Microscope settings:
- Big Ring = 1
- Do not turn fluorescence on
- 10 & 20 objectives for sperm on ph1
- 40x = PH 2
- PH 3 = morphology
- 0 = stained cells only

Freezing Medium: 12% TEST
Refrigeration Medium: 0% TEST

After filling out the EEJ Worksheet and EEJ Data Sheet, staple together and place inside the “CCF EEJ” binder inside the Repro Lab.
REAGENT-CONCENTRATIONS and ORDER Information

Medium for Sperm Collection

All components are found in the clinic fridge or freezer, stock solutions are in the clinic freezer!

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Recipe</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotic Mix</strong></td>
<td>100 µl</td>
<td>1% (v/v)</td>
</tr>
<tr>
<td>10 000 IU/ml Penicillin, 10 mg/ml Streptomycin, 10mg/ml Neomycin Sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fetal Bovine Serum (FBS)</strong></td>
<td>500 µl</td>
<td>5% (v/v)</td>
</tr>
<tr>
<td>(or Fetal Bovine Calf serum if needed)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>L-Glutamine</strong></td>
<td>100 µl</td>
<td>1% (v/v) = 2 mmol/L</td>
</tr>
<tr>
<td>0.2 mol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pyruvic Acid with Sodium Salts</strong></td>
<td>100 µl</td>
<td>1% (v/v) = 10 mmol/L</td>
</tr>
<tr>
<td>1 mol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ham’s F10 Buffer Solution</strong></td>
<td>9 ml</td>
<td></td>
</tr>
<tr>
<td>(9.2 ml if contains Hapes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hepes (if not in Ham’s already)</strong></td>
<td>200 µl</td>
<td>2% (v/v) = 20 mmol/L</td>
</tr>
<tr>
<td>1 mol/L</td>
<td>(none if already in Ham’s)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong>: 10 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

How to make stock solutions:

1. **Antibiotic-Mix**
   - Penicillin/Streptomycin Mix powder, 2 tubes
   - To make up new 10 000 IU/ml penicillin + 10 mg/ml streptomycin solution: add 4.92ml H₂O to the aliquoted powder. The aliquoted powder corresponds to 1/10th of a tube bought from ROTH. Add 0.1g of Neomycin sulfate to this mix. Please check if this is what we have. Solution can be stored for max. 3 days in fridge. Freeze (-20°C) spare aliquots immediately.
   - If we only have a big tube left, separate powder from one tube into 5 parts and add 0.2g of Neomycin sulfate powder. Take one and fill it up to 10 ml with pyrogenfree water. Make 90 aliquots of 110 µl. Freeze them.

2. **L-Glutamine (0.2 mol/L)**
   - M=29.23 g/L
   - ⇒ 0.146 g in 5 ml. Make 45 aliquots of 110 µl. Freeze the aliquots.

3. **Pyruvic Acid with Sodium Salts**
   - M=110.04 g/L
   - ⇒0.55 g in 5 ml. Make 45 aliquots of 110 µl. Freeze the aliquots

4. **Hepes (1 mol/L) - if not in the Ham’s Buffer Solution already**
   - M=238.31 g/L
   - ⇒2.383 g in 10 ml. Make 47 aliquots of 210 µl.
Where to get reagents and disposables:

5. Ham’s F10 Buffer Solution
   Ready to use. Once open it goes bad quickly (store in syringe to prolong shelf life - see p2)
   → check colour and see if it is still clear.
   Irvine Scientific, USA, Ana Lopez [alopez@irvinesci.com], Order No. 99168
   Or more expensive but closer
   IEPSA, South Africa
   Modified Ham’s F-10 Basal Medium 100ml, Catalog# 99175
   Modified Ham’s F-10 Basal Medium 100 ml with HEPES, Catalog#99168

6. TEST
   0%: Irvine Scientific, USA, Ana Lopez [alopez@irvinesci.com], Order No. 90128
   12%: Irvine Scientific, USA, Ana Lopez [alopez@irvinesci.com], Order No. 90129

7. FBS
   Irvine Scientific, USA, Ana Lopez [alopez@irvinesci.com], Order No. 2014

8. Sterile Filters 0.2-0.4µm
   Biodynamics, Bernadette Wells [Bernadette@biodynamics.com.na], SLGV033RS Millex GV
   0.22µm

9. Goblets, Straws and canes
   Veterinary Concepts, USA, Bonnie [sales@veterinaryconcepts.com]
   Straws: e.g. #04166
   Goblets: e.g. #04911
   Canes: e.g. #04916

10. pH-paper
    Ensure it is the correct pH range. Find out where to get it, possibly MEDLAB Angela
    Schaeffler [a.schaeffler@medlabservices.com.na]

11. 1.5ml tubes
    MEDLAB Angela Schaeffler [a.schaeffler@medlabservices.com.na]