The Next Generation Cryo-Preservation System

Protocol for Centrifuging Equine Semen

Just recently, it has become the "La Mode du Reproduction" that all stallions must have their seminal plasma removed in order to ship a dose of semen. In some cases, that is the true reality but in most, it is an added expense of time and labor. Ask yourself "Does my stallion fit the following circumstances"

1. His ejaculate has a large volume although consists of a low <125 million/milliliter sperm count
2. My stallions sperm looks as if it's in shock from the time I collect, until I look at it under a microscope, <10 minutes. This is a result of the seminal plasma being "toxic" to the physical sperm cells; in this situation, the plasma must be removed immediately.

Projected Goal: 200g to 500g for 10 – 15 minutes

1. High Volume/Low Concentration Stallions: Keep "g" force higher towards 500g for 15 minutes

Attempting To Concentrate Your Stallion's Semen

1. Extend at a 1:1 ratio (equal parts of each)
2. Centrifuge @ 750g for 10 minutes
3. Draw off the extender/seminal plasma to ¼" above the sperm pellet
4. Extend semen pellet to 25 million sperm/extended mL

Attempting To Spin Off Your Stallion's Seminal Plasma

1. Keep a decreased "g" force on your centrifuge
2. Extend the entire collection accordingly
    a) You will need to split up the ejaculate in equal volumes in each centrifuge tube
    b) Using a 15 mL tube will allow you to divide up into individual doses
    c) Extend at a 1:1 ratio (equal parts of each)
    d) Centrifuge @ 1000g for 15 minutes
    e) Draw off the extender/seminal plasma to ¼" above the sperm pellet with Pasteur pipette or a 0.25 ml semen straw and syringe or a syringe and "Tom-Cat" catheter
    f) Extend semen pellet to 25 million sperm/extended mL

Using a Centrifugation Cushion

1. It helps greatly to use a centrifugation cushion like the Opti-Prep;
    a) In a 15 mL or 50 mL conical tube
        I. Place 40 ml of extended semen into the centrifuge tube.
        II. Using a 20 mL syringe and a "tom-cat" catheter
        III. Slowly deposit 1 mL (15 mL tube) to 3.5 ml (50 mL tube) of Opti- Prep into the bottom tip of the centrifuge tube
2. Centrifuge @ 1000g for 15 minutes
3. Upon completion, slowly slide the "Tom-Cat" Catheter down the side of the tube, once in the bottom of the cone; slowly draw off the Opti-Prep from the bottom of the tube allowing the sperm pellet to "drop" into the cone of the tube.
4. Then from the top down, slowly aspirate the excess extender/plasma to just above the top of the pellet
5. Extend with fresh extender giving you a end concentration of 25 – 35 million sperm/extended mL

RPM .vs. RCF – Definitions & Setup of Your Centrifuge

RPM - Stands for revolutions per minute -- the speed you set your centrifuge to spin your rotor.

RCF - Stands for relative centrifugal force, also referred to as g-force. The applied force resulting from the spinning action that is perpendicular to the axis of rotation. Force is relative to the earth's gravitational force and has no units, but is simply identified as Xg (times gravity) or RCF.

g - Stands for gravity - a universal constant that represents the natural pull or force of objects towards the earth.

Is there really a difference between RPM and RCF? Yes! Since clean sample separation is dependent on the g-force applied, it is important to run at the proper speed that results in your desired g-force. The speed at which you spin your rotor will affect the amount of force applied to your sample. However, the relationship follows this equation

RCF = 0.00001118 x radius of rotation (cm) x RPM^2

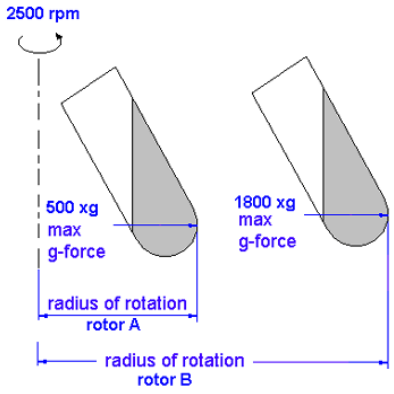
This means that RCF increases exponentially with an increase in RPM. This relationship is shown pictorially below.

How to Calculate the Proper "g" Force for Centrifuging Semen

RPM = $\sqrt{g/0.0000112} * r$ (r = Radial Arm Length)

To measure the Radial Arm Length: measure from the center of the rotor to the tip/end of the centrifuge tube *utilizing millimeters*

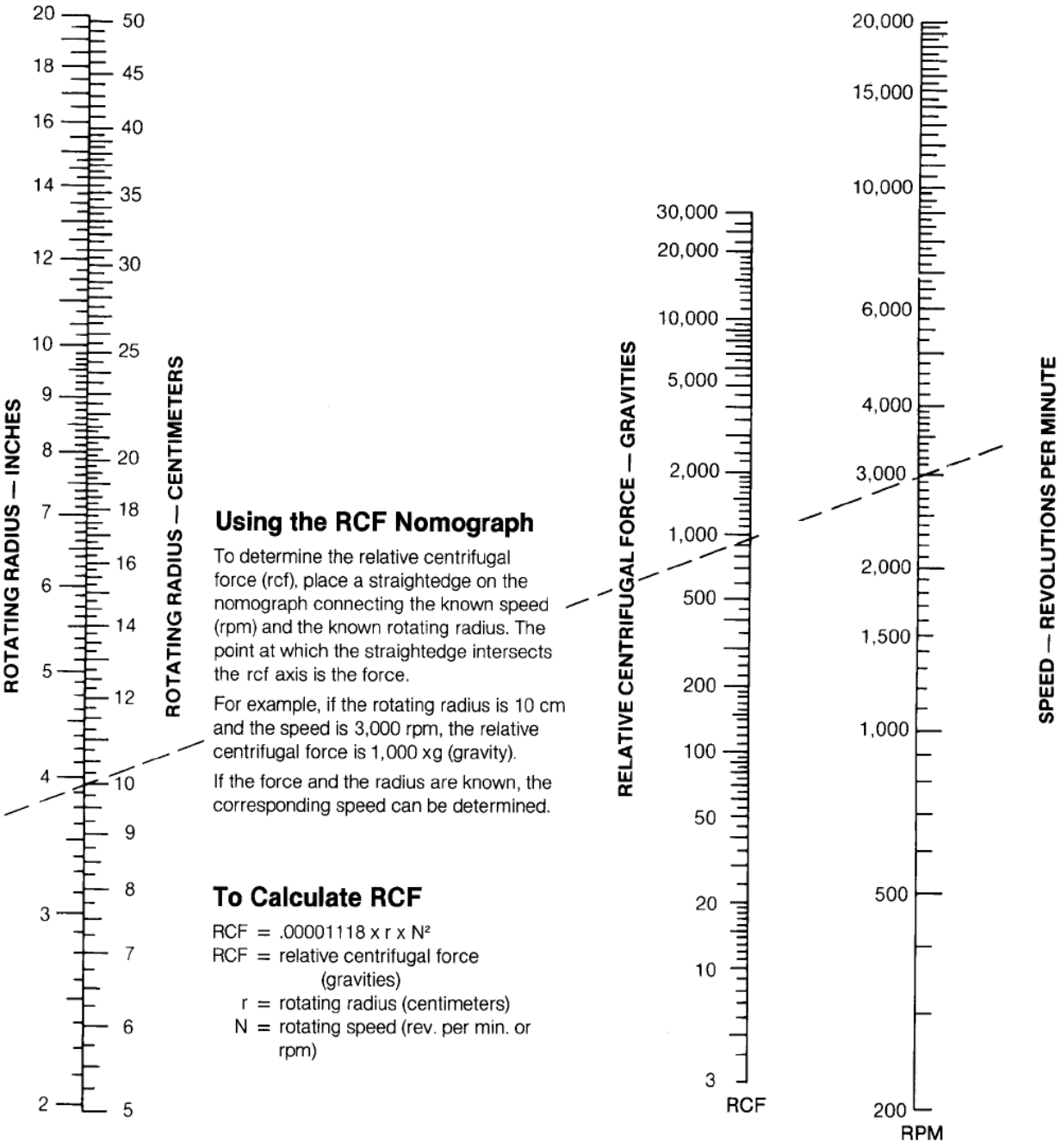
The centrifuge must have a "swing rotor" design



G-Force Calculator Explained

The G-Force Calculator is provided to aid in your centrifuge selection, because RPM and RCF are not the same. G-force is the driving force behind your separation. The speed at which the rotor spins produces a centrifugal force that follows this relationship,

4.5 Nomograph



Using the RCF Nomograph

To determine the relative centrifugal force (rcf), place a straightedge on the nomograph connecting the known speed (rpm) and the known rotating radius. The point at which the straightedge intersects the rcf axis is the force.

For example, if the rotating radius is 10 cm and the speed is 3,000 rpm, the relative centrifugal force is 1,000 xg (gravity).

If the force and the radius are known, the corresponding speed can be determined.

To Calculate RCF

RCF = .00001118 x r x N^2
RCF = relative centrifugal force (gravities)
r = rotating radius (centimeters)
N = rotating speed (rev. per min. or rpm)

Using the Next Generation® Semen Freezing Extenders

The Next Generation® Semen Extenders are universally inter-changeable in that they can be used as the spin down extender as well as the final cryopreservation medium. Simplicity is the basis for all of the Next Generation® Semen Extenders.

The Spin down Procedure

Step #1: Select the Next Generation® semen extender that performs best for your stallion; thoroughly mix and allow to remain at room/ambient temperature for 30 minutes so that all components dissolve into solution. Allow the semen to assume room temperature preferably 20°C so that reverse shock is reduced to a minimum. Once your concentration, motility & usable sperm per milliliter have been calculated, divide the semen equally into each 50 mL centrifuge tube; remember, the centrifuge rotor must remain in balance. Know the exact concentration in each tube, extend at a 1:1 ratio cap and invert slowly three (3) or four (4) times to thoroughly mix the semen with the centrifugation extender. I also use a centrifugation cushion and I recommend Opti-Prep and each tube will require a 2 – 4 mL volume laid/folded slowly into the bottom of each tube and you can easily see the cushion building into the cone. Replace the cap finger tight set in each sleeve and set the centrifuge at 1000g for 12 – 15 minutes (see nemograph on how to properly set the g-force)

Step #2: Carefully remove the centrifuge tube from the rotor sleeve, holding up right, remove the cap and using the syringe with the "Tom-Cat" catheter carefully slide against the wall of the centrifuge tube and into the cushion (Opti-Prep) and slowly draw out the cushion which will then make the sperm pellet fall into the cone of the centrifuge tube. Be careful not to draw any sperm, this is highly visible so you can easily control the flow for which you are drawing off the cushion

Step #3: Now it is time to begin to carefully draw off the seminal plasma/super latent; do not let the "Tom-Cat" catheter dip a ¼" below the fluid line. It is very easy to draw off some of the sperm pellet; even with a shaky hand, you are safe down to ¼" above the sperm pellet. Be careful not to draw any sperm, this is highly visible so you can easily control the flow for which you are drawing off the cushion

Preparing the Cryo-Preservation Medium

Step #1: Measure the exact amount of the remaining extender from what was used from the centrifugation medium. Remove the cap from the bottle of cell culture quality glycerol and draw out exactly 2.5% of the total extender volume left with a 10 ml syringe using an 18-gauge needle; dispense the glycerol into the extender bottle. Replace the cap and invert several times, thoroughly blending the glycerol into the extender, do not shake because foam will accumulate.

Step #2: Carefully crack an egg on the edge of a glass beaker; separating the white from the yolk. Once the white has been totally removed, which can also be done by rolling the yolk on the gauze pad or a Next Generation® Max Flow™ Gel Filter.

Step #3: Calculate 5% of the total extender volume and align your pierce point just over half way to the crown on the side of the yolk. Using a 30 ml syringe and an 14 gauge needle, draw out the required volume of *absolute white free yolk* and dispense into the bottle of extender. Replace the cap and invert several times thoroughly mixing the entire group of ingredients.

Note: Varying concentrations of egg yolk can increase/decrease post thaw motility and longevity; it is very good to experiment with the volume of egg yolk in order to maximize your stallion's freezing fertility; never alter the volume of cell culture glycerol above 3% and/or a 1.5:1 egg yolk/glycerol ratio. Conventional thinking is a 1:1 ratio, although a 2:1 extension ratio can increase your post thaw motility by 20 - 25%. With this in mind, testing your stallion becomes an absolute in order to pin point the most advantageous extension ratios and concentrations of egg. Every time you increase the extension ratio, you reduce the usable sperm concentration/straw.

Step #4: Depending upon which straw size you plan to store and maintain your stallion's semen, the extension ratio & concentration/straw will differ accordingly:

a) .5 ml straw: Attempt to achieve 75 - 100 million usable sperm/straw

b) 5ml straw: Attempt to achieve a 350 - 500 million usable sperm/straw

Step #5: Once your straws have been filled and the wick end securely sealed

a) Using a "bubbler" insert into the unsealed end of the straw, which opens a void after sealing so that a bubble can form in the middle of the straw and thus prevent blow off when thawing?

b) Once you have all of the straws "bubbled" then begin to seal with a clean sealing powder so that all of the straws are uniformly sealed.

c) Once all of the straws are sealed, pick them up in your hand with the bubble facing outward, and shake forward; this will move the bubbles to the center of each straw.

Preparing the Next Generation Cryo-Preservation System

a) Given this will be your first run, only begin with one concentration and extension protocol

b) Pull apart all four (4) trays and set in a line

c) Having your straws, mark two (2) or three (3) for each tray with a 1 – 4 or A – D configuration so that you can identify which tray they sat upon during the freezing process.

d) Remember, the .25 mL & .5 mL (smaller diameter) fit one direction and the 2.5 mL (larger diameter) fit the other direction.

e) After the straws are marked and set accordingly on each tray, stack the trays allowing them to nest into each other.

f) Having your refrigerator pre-set at 8□ C or 40□ F simply set the trays in the refrigerator for 70 minutes using a timer.

g) At 50 minutes, fill the large box with six (6) inches of liquid nitrogen replace the lid tightly and allow the box to self "purge" itself; this will consume upwards to an inch of liquid nitrogen.

h) At 65 minutes, add enough liquid nitrogen to bring the height back to six (6) inches.

i) At 70 minutes, pull the tray set out of the refrigerator, set in the nitrogen box, and replace the lid tightly once again; set your timer again for 70 minutes.

j) At 70 minutes, pull off the lid and then pull apart each tray dropping the straws into the liquid nitrogen and remove the trays so that they are not in the way.

k) After you have identified which is the best height (freezing curve) to freeze your stallion/dog you would normally toss in two (2) goblets and one (1) cane which would hold 20 frozen straws. At this point in the process, allow 5 minutes to arrive at the freezing ambient temperature of -180□ C.

The Next Generation Cryo-Preservation System

The Final Step

a) Have an Equine Express set up with a freezing brick readily accessible for the longevity portion of your test.

b) Most thawing protocols suggest dropping the straws directly into a water bath set at 37□ C; we believe that it is best to target the room/ambient temperature. Reason being, when you pull the straws out of the water bath, they will automatically begin to cool to the room/ambient temperature thus exposing the semen to another reverse shock, which again, can damage the acrosome of the sperm on sensitive stallions.

c) Mark 4 different microscope slides with 1 – 4 or A – D so that they coincide with the tray/straw numbers; have the cover slips ready as well.

d) Using a pair of suture scissors, I simply cut off the straw end that was sealed with the powder thus leaving the wick still intact; the semen will not leak out until the other end is cut.

e) Squeeze a drop of semen from each straw onto its corresponding slide and place the cover slip over the drop of semen and place into the incubator for four (4) – five (5) minutes.

f) While the slides are warming, have four (4) 15 mL centrifuge tubes marked with the corresponding tray/straw numbers so that they coincide with the semen straws and place in the bottom of the Equine Express II Semen transport system. Lie in the mid-plate and place a frozen ice brick on top, replace the lid and seal as you would if you were shipping a dose of semen. On 6 – 12 hour intervals you can easily repeat steps “c” & “e” and continue to evaluate the semen longevity post thaw

g) When evaluating each slide, only pull the slide out that you are examining so that you get the true and actual motility post thaw on a warmed basis. Whichever tray looks best is the height that the stallion/dog will freeze best.

To further your study, it is always best to test various extenders, extension protocols (50^6, 100^6, 150^6 & 200^6/mL) and height above the liquid nitrogen until you pin point the absolute best protocol for your stallion/dog.

Our goal was to make freezing semen a task that anyone with a little laboratory experience can easily achieve; we believe the Next Generation Cryogen Preservation System will change the industry just as the Equine Express II semen & embryo transport system accomplished 13 years ago.