PROTOCOL FOR BRI SERUM SPECIMENS

1. Introduction

The purpose of serum samples is to allow us to test for markers of bone loss in women who lose bone most rapidly. Some markers for bone resorption and bone formation are found in the serum while other markers of bone loss are being developed. We will preserve the samples for several years until the best potential markers have been developed.

We will draw enough blood to get four 1-ml tubes of serum from each participant. Buffy coat specimens will be collected at the same time, if applicable (for African American participants, or original SOF cohort members who did not have buffy coat collected at a previous examination). For participants who require buffy coat collection, please refer to the separate PROTOCOL FOR BRI SERUM AND WHITE CELLS AND BLOTTERS.

2. Equipment

Gloves, disposable, non-sterile
1- ml cryotubes for storage and shipping. (Fisher #12-565-169N), 4 per subject
Horizontal centrifuge
10 ml red-top tubes, non-coated (Fisher #02-683-60), 1 per subject
Vacutainer set-ups; 20 or 21 g needles:
    Needle holder (Fisher #02-665-110)
    Needle (20g- Fisher #02-665-31, 21g- Fisher #02-665-21)
Wooden applicator sticks (Fisher #01-340)
Color cap inserts (white) for the 1 mL cryotubes (Fisher #12-565-242), 4 per subject
Non-self defrosting freezer, -20 C, dry ice
Plastic disposable transfer pipettes (with built in bulbs) (Fisher #13-711-5A)
Cryotube storage boxes (100 cell); Fisher #11-678-24B
100 cell cryotube inserts; Fisher #11-678-24C
Insulated shipping boxes for sending cryotubes to BRI; Fisher #03-529-1

3. Procedures

A. Venipuncture

PUT ON GLOVES

a) Before drawing the blood, a preprinted label showing the participant's ID code should be placed on each vacutainer tube. It is essential to then check the ID code on each tube to ensure that the specimen being collected belongs to the participant. This can best be done by holding the tube next to the ID number on the participant's chart and calling out the number. Then ask the participant to say her name aloud and verify it against the name on the chart.

b) Draw blood from an antecubital vein whenever possible. Use a tourniquet to produce venous distention so that a needle can be inserted. A blood pressure cuff inflated midway between systolic and diastolic blood pressure is most effective and is highly recommended. Do not leave the tourniquet in place for more than 2 minutes. This avoids excessive hemoconcentration. If the 2 minute interval is

exceeded, abandon the arm temporarily and attempt to obtain the specimen from the other arm.

c) Remove tourniquet.

d) Draw blood using the vacutainer system (1 10 mL tubes for BRI). For detailed instructions, use those supplied with the vacutainer tubes. A syringe may be used for participants with veins that are too small or fragile for the vacutainer system.

B. BLOOD PROCESSING - RED TOP TUBES

PUT ON GLOVES

a) Allow the filled red-top tubes to stand at room temperature for 20 - 40 minutes. This procedure is necessary to allow an adequate clot to form.

b) After clot formation and before centrifugation, remove the red-top stoppers and gently free the clot from the sides of the tube with a clean plain wooden applicator stick. Replace the stoppers. Balance the tubes of blood for centrifugation. Use a horizontal centrifuge; angle heads are not satisfactory.

c) Centrifuge the blood for 10 minutes at room temperature at a setting known to yield a relative centrifugal force (RCF) of at least 1000 x g at the bottom of the tubes. The table below gives those combinations of centrifuge speed in revolutions per minute (rpm) and rotating radius (r) that will yield an RCF value of 1000 x g. RPM should be read from a tachometer or rev counter when the centrifuge is normally loaded. Radius (r) is measured in centimeters from the center of the rotor shaft to the bottom of the vacutainer tube when the tube is in a horizontal position.

r (cm)	12	14	16	18	20	22.5	26
rpm	2800	2600	2400	2250	2100	2000	1900

Do not use a brake to slow down the centrifuge.

d) Remove serum from the clot by aspiration with a clean transfer pipet (the clot may sometimes stick to plastic). Use a new pipet for each subject. Transfer the serum into four separate prelabeled (see e. below) cryotubes with white stopper inserts. Fill the cryotubes up to the line that is already marked on the tubes (yields approximately 1 ml per tube). Do not go above this line because expansion space is needed when the serum freezes. If there is not enough serum to fill the tube, fill it as much as possible.

e) Each cryotube should be individually labeled with the ppt ID and filled with serum. Use a pen with permanent ink. "Sharpies" work best. Keep the labeled cryotubes away from solvents such as alcohol or acetone as these will erase the ID code. Before transferring the serum, the vacutainer tube and cryotubes should be held side by side and the numbers read aloud to check that the ID code numbers match. Do not set up production lines of labeled empty cryotubes. This increases the chance of error.

f) If the serum is reddish in color, determine if it is hemolyzed or simply contaminated with red blood cells. One can tell the difference by recentrifuging the vacutainer tube. This will pellet any contaminating red cells and the serum

will clear. If the sample is hemolyzed the red color will remain in the serum. If the patient is still in the clinic, another red-top tube should be obtained. Otherwise, the hemolyzed sample should be processed.

g) Insert the white inserts into the serum cryotube caps. Some caution should be used in capping the cryotubes. Screw the caps on firmly to secure them tightly against the rubber gasket, but do not apply an extreme amount of pressure. To promote rapid freezing, place the cryotubes upright in a footless metal rack that is in contact with a shelf in a -20 C freezer.

h) Blood processing should be completed and tubes placed in cold storage within two hours of collection.

4. Temporary Storage and Shipping

a) After samples have been frozen by placing cryotubes upright on a -20 C shelf (overnight), place cryotubes in ID numerical order into a storage box using the inserts.

b) Use the cryotube storage box grid for recording the position of cryotubes, by ID number, within the shipping boxes sent to BRI. (This is a backup identification system in case the ID numbers on the tube are obliterated after prolonged storage at -180 degrees). As the filled tubes are placed into the slots formed by inserts, write the ID number which is on the tube into the corresponding box on the paper grid.

c) Since the box does not have a definite up or down, right or left, you will have to mark the upper right corner of the cardboard box and the insert. (The paper grid is already marked "upper right" and "upper left".) In a clearly visible spot in the upper right corner of the box and the insert (to the right and away from you), punch a hole in the cardboard with a single hole paper punch.

d) Store samples at -20C in the storage box until 100 cryotubes have been filled and frozen. This should take about four weeks.

e) When the box is ready for shipping, record on the grid form the dates over which the samples were collected, your clinic, the date the box was shipped to BRI and number of tubes being shipped.

f) Send one copy of the form to BRI with the box and keep one copy yourself.

g) When the box arrives at BRI, it will be assigned a unique identifier and placed into storage. A copy of the grid with the identifier will be sent to the coordinating center.

h) A box of samples should be shipped in an insulated shipping box on dry ice by a 24 hour air carrier (such as Fed Ex). To ensure that the samples can be received at BRI the next day, ship Monday - Wednesday only. The insulated shipping box and method can be chosen by each center. However, the shipping box must provide insulation and have inner dimensions sufficiently large to handle 1 storage box (o.d. 5.25 x 5.25.x 4.75 inches) and 5-10 lbs of chipped dry ice to keep the samples cold even if shipment is interrupted for a day or two. (If in doubt, err on the side of too much dry ice.)

i) Serum should be shipped to:

Don Dover
Biomedical Research Institute
12264 Wilkins Ave
Rockville, Maryland 20852

5. Summary of Important Rules

a) The tourniquet must not be in place for more than 2 minutes.

b) The tourniquet must be released before blood is drawn.

c) Vacutainer tubes and cryotubes must be pre-labeled with the participant's ID code number. Vacutainer tube ID numbers must be checked with the participant's chart immediately before venipuncture. Cryotube ID numbers must be checked with each respective vacutainer tube before transferring the samples.

d) The red-top vacutainer tubes must be kept at room temperature for at least 20 miuntes but no longer than 40 minutes before centrifugation.

e) Blood processing should be completed and serum stored in the freezer within 2 hours of collection.

PROTOCOL FOR BRI SERUM, WHITE CELLS AND BLOTTERS

I. SERUM AND WHITE CELLS

1. Introduction

This protocol should be used for all participants who have not previously had buffy coat specimens collected.

The purpose of serum samples is to allow us to test for markers of bone loss in women who lose bone most rapidly. Some markers for bone resorption and bone formation are found in the serum while other markers of bone loss are being developed. We will preserve the samples for several years until the best potential markers have been developed.

We will draw enough blood to get four 1-ml tubes of serum from each participant. Buffy coat specimens will be collected at the same time

2. Equipment:

Gloves, disposable, non-sterile
Horizontal centrifuge
Non-self-defrosting freezer, -20°C dry ice
8.5 ml yellow top vacutainer tubes (Fisher #02-684-26),
containing acid citrate dextrose (ACD)-two per participant
4 ml cryotubes-one per participant, Fisher #12-565-161N
Vacutainer set-ups; 20 or 21 g needles:
Needle holder (Fisher #02-665-110)
Needle (20g- Fisher #02-665-31, 21g- Fisher #02-665-21)
10 ml red-top tubes, non-coated (Fisher #02-683-60), 1 per subject
1 ml cryotubes-four per participant (Fisher #12-565-169N)
Color cap inserts (white) for the 1 mL cryotubes (Fisher #12-565-242), 4 per subject
Color cap inserts (yellow) for the 4 ml cryotybes (Fisher #12-565-246), 1 per subject
Wooden applicator sticks (Fisher #01-340)
10 cc syringes-one per participant
Plastic disposable transfer pipets (with built-in bulbs)
Cryotube storage boxes (100 cell); 100 cell cryotube inserts
Insulated shipping boxes for sending cryotubes to BRI
Sterile normal saline (a liter should last a long time; keep refrigerated)

3. Procedures:

A. Venipuncture

PUT ON GLOVES

a) The participant must have been seated for 10 minutes before venipuncture. This standardizes the degree of orthostatic hemoconcentration.

b) Before drawing the blood, a preprinted label showing the participant's ID code should be placed on each vacutainer tube. It is essential to then check the ID code on each tube to ensure that the specimen being collected belongs to the participant. This can best be done by holding the tube next to the ID number on the participant's chart and calling out the number. Then ask the participant to say her name aloud and verify it against the name on the chart.

c) Draw blood from an antecubital vein whenever possible. Use a tourniquet to produce venous distention so that a needle can be inserted. A blood pressure cuff inflated midway between systolic and diastolic blood pressure is most effective and is highly recommended. Do not leave the tourniquet in place for more than 2 minutes. This avoids excessive hemoconcentration. If the 2 minute interval is exceeded, abandon the arm temporarily and attempt to obtain the specimen from the other arm.

d) Remove tourniquet.

e) Draw blood using the vacutainer system (1 10 mL red-top tube Fisher #02-683-60; and two yellow-top 8.5 ml, Fisher #02-684-26). For detailed instructions, use those supplied with the vacutainer tubes. A syringe may be used for participants with veins that are too small or fragile for the vacutainer system.

f) Draw yellow-top first, as close to two full tubes as possible.

g) Gently turn the yellow-top tubes a few times to allow the anticoagulant to mix with the blood.

B. BLOOD PROCESSING - RED TOP TUBES

PUT ON GLOVES

a) Allow the filled red-top tubes to stand at room temperature for 20 - 40 minutes. This procedure is necessary to allow an adequate clot to form.

b) After clot formation and before centrifugation, remove the red-top stoppers and gently free the clot from the sides of the tube with a clean plain wooden applicator stick. Replace the stoppers. Balance the tubes of blood for centrifugation. Use a horizontal centrifuge; angle heads are not satisfactory.

c) Centrifuge the blood for 10 minutes at room temperature at a setting known to yield a relative centrifugal force (RCF) of at least 1000 **x** g at the bottom of the tubes. The table below gives those combinations of centrifuge speed in revolutions per minute (rpm) and rotating radius (r) that will yield an RCF value of 1000 **x** g. RPM should be read from a tachometer or rev counter when the centrifuge is normally loaded. Radius (r) is measured in centimeters from the center of the rotor shaft to the bottom of the vacutainer tube when the tube is in a horizontal position.

r (cm)	12	14	16	18	20	22.5	26
rpm	2800	2600	2400	2250	2100	2000	1900

Do not use a brake to slow down the centrifuge.

d) Remove serum from the clot by aspiration with a clean transfer pipet (the clot may sometimes stick to plastic). Use a new pipet for each subject. Transfer the serum into

four separate prelabeled (see e. below) cryotubes with white stopper inserts. Fill the cryotubes up to the line that is already marked on the tubes (yields approximately 1 ml per tube). Do not go above this line because expansion space is needed when the serum freezes. If there is not enough serum to fill the tube, fill it as much as possible.

e) Each cryotube should be individually labeled with the ppt ID and filled with serum. Use a pen with permanent ink. "Sharpies" work best. Keep the labeled cryotubes away from solvents such as alcohol or acetone as these will erase the ID code. Before transferring the serum, the vacutainer tube and cryotubes should be held side by side and the numbers read aloud to check that the ID code numbers match. Do not set up production lines of labeled empty cryotubes. This increases the chance of error.

f) If the serum is reddish in color, determine if it is hemolyzed or simply contaminated with red blood cells. One can tell the difference by recentrifuging the vacutainer tube. This will pellet any contaminating red cells and the serum will clear. If the sample is hemolyzed the red color will remain in the serum. If the patient is still in the clinic, another red-top tube should be obtained. Otherwise, the hemolyzed sample should be processed.

g) Insert the white inserts into the serum cryotube caps. Some caution should be used in capping the cryotubes. Screw the caps on firmly to secure them tightly against the rubber gasket, but do not apply an extreme amount of pressure. To promote rapid freezing, place the cryotubes upright in a footless metal rack that is in contact with a shelf in a -20 C freezer.

h) Blood processing should be completed and tubes placed in cold storage within two hours of collection.

C. PROCESSING THE YELLOW-TOP TUBES

PUT ON GLOVES.

a) Blood in the yellow-top tubes will not clot, but may settle. This is not a problem.

b) The buffy coat from both yellow-top tubes for each participant will be placed in the same cryotube. **Be extremely careful that both buffy coats from each participant are placed in the same cryotube and buffy coats from different participants are not mixed.**

c) Centrifuge tubes at approximately 2000 rpm for 20 minutes. Do not brake.

d) Label a 4 cc cryotube with the SOF ppt ID. Use a pen with permanent ink such as a "Sharpie." Keep the labeled cryotubes away from solvents such as alcohol or acetone as these will erase the ID code. Before transferring the buffy coat, the yellow-top tube and the cryotube should be held side by side and the numbers read aloud to check that the ID code numbers match. Do not set up production lines of labeled empty cryotubes. The chance of error is increased by the latter procedure.

e) After centrifuging, the sample should be in three layers in tube: bottom = red blood cells; middle = buffy coat (white blood cells); top = plasma. The buffy coat will be much smaller than the other two layers.

f) Using a plastic pipet, remove and dispose of the top layer of plasma. Begin near the top, and work your way down. Stop just above the buffy coat, leaving a little plasma behind.

g) Gently swirl the tube to loosen the buffy coat. Pipet out the buffy coat, using a plastic pipet, with the remaining plasma. Place the buffy coat into a 4 ml cryotube. Insert the yellow insert into the buffy coat cryotube cap.

h) Repeat the previous three steps with the participant's second yellow-top tube. **Be extremely careful that both buffy coats from each participant are placed in the same cryotube and buffy coats from different participants are not mixed.**

i) Place cryotubes upright in a footless rack in contact with shelf in -20°C freezer.

D. SOLUTIONS FOR SOME COMMON PROBLEMS

- If unable to obtain a full 8.5 cc's of blood for the yellow-top tubes, obtain as much as possible and process what you have.
- If there are a few red cells in the buffy coat sample, this is all right. However, as few as possible is preferable.
- If there are a lot of red cells in the buffy coat or the buffy coat "sticks" to the red cells: 1) recentrifuge the sample and try to pipet the buffy coat off again; 2) if it is still a problem, add about 5 ml of sterile normal saline to the yellow top tube and recentrifuge. Then pipet off top layer (which will now be the saline) and then pipet the buffy coat off as above.
- If there isn't a buffy coat layer after centrifuging, place tube back in the centrifuge and spin for another 10 minutes. If there still isn't a buffy coat layer, but other tubes in the same batch did layer, save the lowest portion of the plasma layer. If there isn't a buffy coat layer after the second centrifuging and other tubes in the same batch did not layer either, there is probably something wrong with the centrifuge. Save specimens at room temperature for up to 72 hours while fixing/replacing.

E. TEMPORARY CRYOTUBE STORAGE AND SHIPMENT

- After samples have been frozen by placing cryotubes upright on a -20°C shelf overnight, place cryotubes in ID numerical order into a storage box using the inserts.
- Use the cryotube storage box grid for recording the position of cryotubes, by ID number, within the shipping boxes sent to BRI. (This is a back-up identification system in case the ID numbers on the tube are obliterated after prolonged storage at -70°C.) As the filled tubes are placed into the slots formed by inserts, write the ID number which is on the tube into the corresponding box on the paper grid. Write a dash and the letter W or Y after the ID to record whether the tube cap contained a white (serum) or yellow (buffy coat) insert.
- Since the box does not have a definite up or down, right or left, you will have to mark the upper right corner of the cardboard box and the insert. (The paper grid is already marked "upper right" and "upper left."). In a clearly visible spot in the upper right corner of the box and the insert (to the right and away from you), punch a hole in the cardboard with a single hole paper punch.

- Store samples at -20°C in the storage box until 25 cryotubes have been filled and frozen.

- When the box is ready for shipping, record on the grid form the dates over which the samples were collected, your clinic, the date the box was shipped to BRI, and the number of tubes being shipped.

- Send one copy of the form to BRI with the box and keep one copy yourself.

- When the box arrives at BRI it will be assigned a unique identifier and placed into storage. A copy of the grid with the identifier will be sent to the coordinating center.

- A box of samples should be shipped in an insulated shipping box on dry ice by a 24-hour air carrier (such as Federal Express). To ensure that the samples can be received at BRI the next day, ship Monday-Wednesday only. The insulated shipping box and carrier can be chosen by each Center. However, the shipping box must provide insulation and have inner dimensions sufficiently large to handle 1 storage box (outside dimensions 5.25 x 5.25 x 4.75 inches) and 5-10 lbs of chipped dry ice to keep the samples cold even if shipment is interrupted for a day or two. (If in doubt, err on the side of too much dry ice.)

- Serum should be shipped to:

  Don Dover
  Biomedical Research Institute
  12264 Wilkins Ave., Bay E
  Rockville, Maryland 20852
  301/881-4513
  301/770-9811 (F)

II. WHOLE BLOOD ON BLOTTERS FOR PCR ANALYSIS

1. Equipment:

   Filter paper: Whatman BFC180 3 by 3 inch bloodstain cards, 1 per participant
   Drierite: 5lbs Indicator Mesh #8
   40 ZipLoc™ plastic bags (food storage size)
   Airtight plastic container for storage of ZipLoc™ bags

2. Procedures:

   a) Label the corner of a 3 in. by 3 in. piece of filter paper with the subjects ID and namecode, using a #2 pencil.

   b) Before spinning, draw about ½ cc whole blood from a yellow-top tube with a plastic pipette. If blood has settled, mix thoroughly before pipetting.

   c) Decant the ½ cc of whole blood onto the filter paper. Hold up a corner of the filter paper while decanting so that the blood doesn't soak through to the paper towel. The Whatman bloodstain cards will have four circles intended for use as a reference. However, the blood can simply be dropped into the center of the card, and need not be applied within the circles.

   d) Place the whole blood/filter paper on a paper towel and allow to air dry.

   e) When the blood is completely dry, place the numbered filter paper in a ZipLoc™ plastic bag. The plastic bag should contain 2 tspn Drierite. Store about 50 pieces of filter paper in each plastic bag, clipped (plastic clip) together in ID order. Record the ID numbers contained in each ZipLoc™ bag on a piece of paper taped to the insider of the bag and visible from without.

   f) Store the plastic bags in the airtight plastic container along with 1 Tbsp Drierite for each bag. Store the plastic container in the refrigerator.

   g) Monitor the Drierite in the baggies and the plastic container each week. If the Drierite changes from blue to pink-red in color, replace it with a fresh sample. The Drierite may be recycled by warming it in an oven until it turns blue again.

   h) The entire batch of specimens will be shipped to BRI once all Year 12 participants have been completed.