

Interactive Medical Technology®

ULTRASPHERS™ MICROSPHERE EXTRACTION PROTOCOL FOR REGIONAL BLOOD FLOW MEASUREMENT

NO. 91001.4

PROCEDURAL INSTRUCTIONS

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FOR TECHNICAL ASSISTANCE

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INTRODUCTION

E-Z TRAC Ultraspheres™ (Colored Microspheres) provides the research scientist with an alternative to the use of radioactive microspheres for the measurement of blood flow in experimental animals. The high equipment and disposal costs normally associated with radioactive microspheres are eliminated with the E-Z TRAC non-radioactive colored microspheres. The minimum required laboratory equipment consists of minimal glassware, a multipurpose centrifuge, a hemacytometer counting slide and a microscope. For laboratories requiring high productivity alternatives, E-Z TRAC offers the TRACker 1000™, a microscopic optical imaging workcenter for **automated counting and analysis** of colored microspheres and **on-line Regional Blood Flow calculation**.

The colored microspheres prepared by E-Z TRAC are crosslinked polystyrene-divinylbenzene microspheres which have been labeled with eight distinct colors: red, blue, orange, green, yellow, coral red, violet and black. The high dye content of each individual microsphere allows rapid identification and discrimination under microscopic visualization. The colored microspheres are chemically stable and exhibit no dye leaching in aqueous environments, including strong acid and base solutions. Each color of microsphere is available in a wide range of sizes, from 5 µm diameter to 200 µm diameter.

The method of animal preparation and microsphere injection with the E-Z TRAC colored microspheres are the same as that followed with radioactive microspheres in blood flow studies. To measure regional myocardial blood flow, for example, the microspheres are injected into the left atrium of the experimental animal. The E-Z TRAC colored microspheres will mix with the arterial blood and flow with it, will distribute to the tissue, and will ultimately lodge in the microvasculature. The number of microspheres in the myocardial tissue of interest and in reference blood sample are determined by following an extraction procedure using reagents and instructions supplied by E-Z TRAC.

The feasibility of non-radioactive microspheres for blood flow research has been demonstrated by previous studies using black non-radioactive microspheres and alkaline hydrolysis of tissue and blood samples. The lack of additional colors limited these studies to only one blood measurement for a single diameter of microsphere. Employing the multiple colors of E-Z TRAC microspheres, blood flow studies may now consist of up to seven sequential measurements. Simultaneous blood flow measurements with 11.1 mm diameter radioactive microspheres and 11.9 mm diameter E-Z TRAC colored microspheres following coronary occlusion have shown a correlation in the measurement of regional myocardial blood flow of $r = 0.98$. With the availability of E-Z TRAC non-radioactive microspheres in seven colors and a range of sizes, institutions are no longer prevented from conducting such research.

EQUIPMENT AND PLASTICWARE REQUIRED BUT NOT SUPPLIED

Hemacytometer (2-4 recommended)

Fuchs-Rosenthal ruling and/or Improved Nuebauer ruling, (Hausser Scientific, Horsham, PA 19044)

15 ml Graduated Polypropylene Conical Centrifuge Tubes with screw caps

50 ml Graduated Polypropylene Conical Centrifuge Tubes with screw caps

Glass Disposable Pasteur Pipettes

Centrifuge

Balance

Microscope, 100x - 400x Magnification (or E-Z TRACker 1000)

Boiling Water Bath

Thermolyne Maxi-Mix vortex mixer

5-key Blood Cell Counter or Hand-held Counter

Ultrasound Homogenizer (recommended)

General Purpose Oven

REAGENTS REQUIRED FOR TISSUE AND BLOOD PROCESSING

1. TISSUE/BLOOD DIGEST REAGENT I: (Catalog No. 501-105) 2X Concentrate, 250ml. Before use, dilute bottled reagent two-fold by the addition of 1 volume reagent to 1 volume distilled water. **CAUTION: Strong Alkaline Solution!** Wear gloves and handle with care. Avoid exposure to the skin, eyes and clothing.

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2. TISSUE/BLOOD DIGEST REAGENT II: (Catalog No. 501-106) 5X Concentrate, 250ml. Before use, dilute bottled reagent five fold by the addition of 1 volume reagent to 4 volumes distilled water.
3. MICROSPHERE COUNTING REAGENT: (Catalog No. 501-107) 5X Concentrate, 250ml. Before use, dilute bottled reagent five fold by the addition of 1 volume reagent to 4 volume distilled water.
4. BLOOD HEMOLYSIS REAGENT: (Catalog No. 501-108) 5X Concentrate, 250ml. Before use, dilute bottled reagent five fold by the addition of 1 volume reagent to 4 volume distilled water.
5. LIPID EMULSIFIER REAGENT: (Catalog No. 501-109) 1 Liter. Special reagent for processing fatty tissue specimens (e.g., brain, spinal cord). No dilution required.

Regional Blood Flow Experimental Protocol Recommendations

Regional organ perfusion can be estimated with hematogenously delivered microspheres (Heyman et al., 1997). When appropriately sized microspheres are used, regional blood flow is proportional to the number of microspheres trapped in the region of interest (Bassingthwaight et al., 1990) a number of excellent review article describe and validate the use of microspheres for measurement of region blood flow, but the classic review by Heyman et al. (1977) contains many detail for radioactive microspheres use that apply to colored and fluorescent microspheres (Glenny et al., 1999).

Over the last twenty years, new and/or refined methods for measuring regional blood flow have been published. A careful search of the literature should be done prior to starting a study to determine the most appropriate method for measuring regional organ perfusion for any given experiment protocol. It is not feasible for this protocol to address the wide variety of methods currently accepted. Outlined below are methods for regional blood flow that have been recommended by our customers and the Fluorescent Microsphere Resource Center (Glenny et al., 1999).

Calculation of Microspheres for Injection

A minimum of 400 microspheres are needed per tissue piece to be 95% confident that the flow measurement is within 10% of the true value (Buckberg et al., 1971). If measurement of regional blood flow (ml/min) is the primary objective of a study, the number of microspheres must be calculated to assure a sufficient number reach the organ of interest.

The following equation estimates the minimum total number of microspheres needed per injection to accurately measure blood flow.

$$N_{\min} = 400(n)/[Q_{\text{organ}}/Q_{\text{total}}]$$

Where:

N_{\min} = minimum number of microspheres needed for injection

n = total number of organ pieces

Example: In a 200-gram heart, if you plan to evaluate ten, 1-gram pieces, the total number of pieces per organ should be 200 (even though you plan to evaluate only 5% of the organ).

$[Q_{\text{organ}}/Q_{\text{total}}]$ = fraction of the total cardiac output supplying the organ of interest

With this calculation, 400 microspheres are provided for each organ piece with an average blood flow. A piece with twice the average flow will have 800 microspheres and a piece with 0.5 times the average flow will have 200

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microspheres. Doubling the minimum number (N_{\min}) will make sure that low-flow organ pieces also have adequate number of microspheres (Buckberg et al., 1971; Nose et al., 1985). This 400 microsphere “rule” only applies to measurement of perfusion to a single region or organ piece. A recent paper by Polissar et al, 1999, reports that fewer microspheres are required for accurate measurements of heterogeneity and correlation. Polissar suggests a minimum of 15,000 microspheres, total, for all pieces combined for accurate measurements of heterogeneity and 25,000 microspheres, total, for accurate estimated of correlation coefficients.

Calculation of Injectate Volume

$$\text{mls of suspension} = N_{\min} / [\text{microspheres concentration}]$$

Preparation of Ultraspheres™ for Injection

Method:

1. Check supernatant solution. It should be clear due to the addition of thimerosal, a bacteriostatic agent. Cloudy fluid may indicate contamination. The fluorescent microsphere suspensions contain a small amount of Tween 80 (0.050% v/v) to prevent microsphere aggregation. If desired, the concentrations of additives can be reduced by centrifuging the suspension, aspirating the supernate and diluting the microspheres with the desired carrier containing a minimum 0.01% (v/v) Tween 80 just prior to injection.
2. Mix by inverting slowly and thoroughly (15 to 30 sec). (Note: Vortexing microspheres can create foam that will cause variations in microsphere concentration.)
3. Place in ultrasonic water bath for at least 2-10 min to disperse the microspheres (Recommended, but not required). Do not cover water bath or sonicate too long because the heat generated can melt the microspheres. For microspheres 15 μ or smaller, sonication time is less (smaller particles are more susceptible to heat).
4. Just prior to injection, invert mix the vial of microspheres again, load and unload the syringe to the desired volume three times. Do not permit the microspheres to settle once they are drawn into the syringe. If injection time is delayed, repeat the loading procedure.
5. Injection time varies for each experimental design and must be determined prior to injection. Most left-heart injections are done over short periods of time (5-15 seconds) while the reference withdrawal pump is withdrawing the sample. A left-heart injection should not appreciably change the animal's stroke volume. Slow and steady injections allow for uniform mixing of microspheres, whereas bolus injections often result in streaming (not desirable). Following injection, a visible rim of microspheres will remain in the syringe if plastic syringes are used. This can be expected and is of little concern since the remaining microspheres represent only a small fraction of the total used.
6. After injection, flush the dead space of the catheter thoroughly (at least three times the volume of the catheter) and change the stopcock (microspheres get caught in the stopcocks; Discard along with the used syringes after each injection to avoid contamination of subsequent injections).

Reference Blood Flow Sampling

A reference blood flow sample allows calculation of regional flow in ml/min. It is essential that the reference blood withdrawal catheter be accurately positioned so that a representative sample of well-mixed microspheres and blood can be obtained. If pulmonary perfusion is being measured, the reference blood sample should be obtained from a pulmonary artery. If systemic organ flows are measured, reference blood samples can be obtained from the descending aorta. The blood samples should be obtained as close to the organ of interest as possible without interfering with blood flow.

The site of microsphere injection is very important. For systemic blood flow measurements, left atrial injection of

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microspheres is best. If a left atrial catheter is not possible, then a left ventricular catheter is adequate. Left atrial injections allow for two-chamber mixing and more uniform distribution of the microspheres in the blood. Left ventricular injections allow one chamber mixing, shown to be sufficient in most species.

The reference withdrawal pump must be accurately calibrated so that reference blood is withdrawn at a uniform rate. If problems exist in the rate of withdrawal, the reference sample is invalid. Whenever possible, two reference blood samples should be withdrawn simultaneously, in case one catheter clots or one sample is lost.

Withdrawal syringes must be large enough to hold the volume of blood in the reference sample, heparin, and dead space volume. Example: if withdrawal rate is 5 ml/min and withdrawal time is 2 min after completion of a 1-min injection, heparin volume is 1 ml and dead space is 3 mls, then the syringe volume should be 20-30 mls. Glass syringes and containers are preferred; they decrease microsphere loss caused by "static" attraction of the plastic microspheres with the plastic containers or syringes.

Method for Reference Blood Flow Sampling

There are 3 different anticoagulants that routinely used: Heparin (syringe coated), Citrate Phosphate Dextrose (10 cc per 30 cc syringe) and EDTA*Na₂(2mg/ml of blood).

1. Using whole blood, calibrate the reference withdrawal pump at the predetermined withdrawal rate, including the catheters, extension tubing and matched anticoagulated glass syringes that will be used for the reference withdrawal. Have new stopcocks and flush syringes available.
2. Connect the matched glass anticoagulated syringes in the withdrawal pump to the catheters and the extension tubing so that everything is set up for withdrawing the reference sample. Do not turn the stopcock on the catheters until you are ready for injection (the blood will flux into the catheter dead space and may clot).
3. Once the microspheres have been drawn into the injection syringes, start the withdrawal pump and make sure blood is flowing freely into the extension tubing. 4. Inject the microspheres over the designated time period (sec or min) followed by a flush of warmed saline three times the volume of the catheter dead space.
4. A timer is set for 2 min after completion of injection for the reference blood withdrawal. At the end of the withdrawal, the pump is turned off, the stopcocks are opened and the blood remaining in the extension tubing is drawn into the syringe.
5. Transfer blood into labeled test tubes for further processing (see Digestion of Blood and Tissue, next page). Rinse syringes and extension lines with 2% Tween-80 ® (using approximately twice the volume of the blood) and add this rinse to the blood samples.
6. Flush the catheters again and change the stopcocks.

RECOVERY OF MICROSPHERES FROM BLOOD AND TISSUE

GENERAL NOTES

1. Tissue biopsy samples should be refrigerated (less than 1 week) or stored frozen or fixed with formaldehyde until such time as one proceeds with the following extraction procedures. Reference blood samples should be stored refrigerated (Make sure enough anticoagulant is mixed in with the blood).
2. Before mixing by vortex or inversion, all centrifuge tubes should be covered with a screw cap to prevent spillage and loss of microspheres.
3. Unless otherwise noted, all procedures are performed at room temperature.
4. Due to the wide variety of animals and tissue organ types some adjustment may be necessary to dissolve the fatty tissue organs.

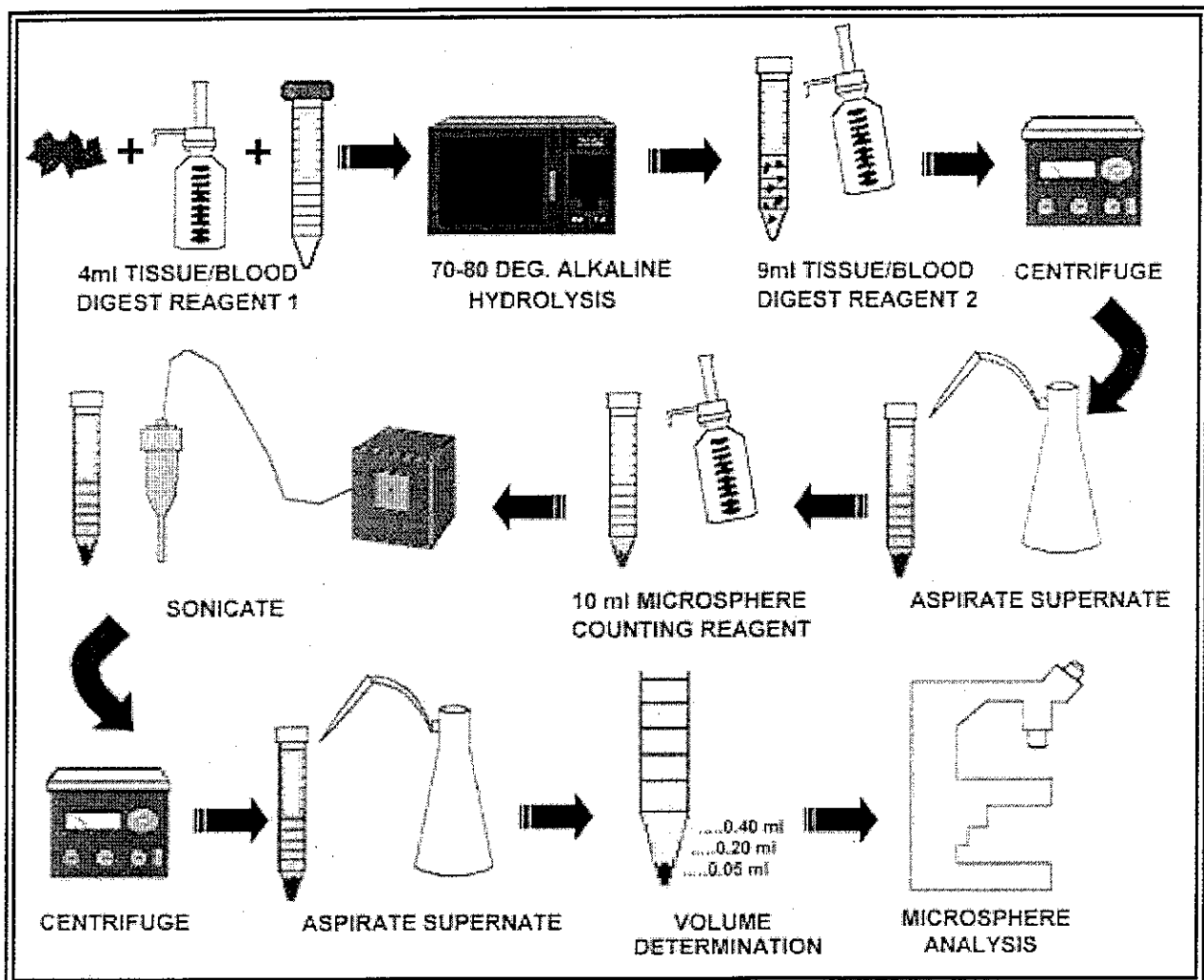
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- Unless otherwise noted, all centrifugation steps are at a RCF of 1500 x g with low or no brake (approximately 2,500 – 3,000 RPM for common bench top centrifuges).
- Unless other wise notes, all 15 ml sample tubes should be sonicated with a 2 mm stepped micro tip and 50 ml tubes with a 1/8" micro tip. Power should be adjusted by starting with a low setting and increase until the pellet begins to break up. Maintain this power setting for the remainder of the samples.

RECOVERY OF MICROSPHERES FROM TISSUE BIOPSY SAMPLES

Two similar procedures may be used for processing tissue biopsy samples. The sample processing steps for the first procedure are performed in a 15 ml centrifuge tube and is recommended for most organ tissue samples of 1-3 grams. The second procedure is recommended for tissue samples greater than 3 grams or for tissue sample that prove difficult to hydrolyze with the first procedure.

RECOVERY OF MICROSPHERES FROM TISSUE SAMPLES



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PROCEDURE I.

RECOVERY OF MICROSPHERES FROM TISSUE SAMPLES (less than 3 gram)

1. Each whole weighed tissue sample (<3 gram) is placed in a 15 ml polypropylene centrifuge tube and pushed or centrifuged to the bottom.
2. 4 ml of diluted TISSUE/BLOOD DIGEST REAGENT I (****CAUTION*** Strong Alkaline Solution) is added to each sample. Place the screwcaps on the centrifuge tubes and snugly tighten.
3. Either of the following procedures may be employed during the alkaline hydrolysis of the tissue samples:
 - I. **Overnight Tissue Hydrolysis:** Place the tubes in a general purpose oven {Preferred} or heated water bath at 70°-80°C and allow the tissue to hydrolyze overnight. (CAUTION: Cover the water bath; with a lid to prevent the evaporation of the heated water.) The following day, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. The tissue samples in each tube should completely homogenize into suspension, with only small particles of fatty white debris visible.
 - II. **Same Day Tissue Hydrolysis:** Place the tubes in a heated water bath (90°-100°C). The level of the water bath should be even with that of the sample and maintained by periodic additions. Following 15 min. of heating, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. A major portion of the tissue samples in each tube should homogenize into suspension. Return the centrifuge tubes to the boiling water bath for further heating. At periodic intervals (5-10 min.), remove the centrifuge tubes from the water bath and vigorously vortex as described above. This is repeated until all of the tissue is homogenized into suspension and only small particles of fatty white debris are visible. Return the centrifuge tubes to the boiling water bath for a final 15-30 min. of heating. Following the alkaline hydrolysis of the tissue samples, proceed immediately to next step, fats and lipids in the sample may gel upon standing.
4. Add diluted TISSUE/BLOOD DIGEST REAGENT II to the sample suspension to bring the total liquid volume to 14-15 ml. Cover with the screw-cap and mix by inversion. Centrifuge the tubes for 30 min. and aspirate the supernate to a level slightly above each pellet.
5. Each greenish-brown sediment is re-suspended in 10 ml of diluted MICROSPHERE COUNTING REAGENT by vortex mixing. If small sediment aggregates are visible, they can be disrupted by either (A) brief ultrasound sonication for 15-30 seconds {Recommended}, or (B) returning the centrifuge tubes to the oven/boiling water bath for 15 min. and repeating the combination of heating and vortexing until all large aggregates are dispersed.
6. Centrifuge for 15 min., and aspirate the supernate to a level slightly above the visible pellet. Briefly centrifuge the tube to sediment any liquid on the centrifuge wall or cap. Proceed to DETERMINATION OF MICROSPHERES SAMPLE VOLUME.

PROCEDURE II.

RECOVERY OF MICROSPHERES FROM LARGE TISSUE SAMPLES (3-9 gram)

1. Each whole weighed tissue sample (3-9 gram) is placed in a 50 ml centrifuge tube and pushed or centrifuged to the bottom.
2. 15 ml of diluted TISSUE/BLOOD DIGEST REAGENT I (****CAUTION*** Strong Alkaline Solution) is added to each sample. Place the screwcaps on the centrifuge tubes and snugly tighten.
3. Either of the following procedures may be employed during the alkaline hydrolysis of the tissue samples:
 - I. **Overnight Tissue Hydrolysis:** Place the tubes in a general purpose oven {Preferred} or heated water bath at 70°-80°C and allow the tissue to hydrolyze overnight. (CAUTION: Cover the water bath with a lid to prevent the evaporation of the heated water.) The following day, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. The tissue samples in each tube should completely homogenize into suspension, with only small particles of fatty white debris visible.
 - II. **Same Day Tissue Hydrolysis:** Place the tubes in a heated water bath (90°-100°C). The level of the water bath should be even with that of the sample and maintained by periodic additions. Following 15 min. of heating, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. A major portion of the tissue samples in each tube should homogenize into suspension. Return the centrifuge tubes to the boiling water bath for further heating. At periodic

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intervals (5-10 min.), remove the centrifuge tubes from the water bath and vigorously vortex as described above. This is repeated until all of the tissue is homogenized into suspension and only small particles of fatty white debris are visible. Return the centrifuge tubes to the boiling water bath for a *final 15-30 min. of heating. Following the alkaline hydrolysis of the tissue samples, proceed immediately to next step, fats and lipids in the sample may gel upon standing.*

4. Add diluted TISSUE/BLOOD DIGEST REAGENT II to the sample suspension to bring the total volume to 45-50 ml. Cover with the screw-cap and mix by inversion. Centrifuge the tubes for 30 min. and aspirate the supernate to a level slightly above each pellet.
5. Each greenish-brown sediment is re-suspended in 5 ml of diluted MICROSPHERE COUNTING REAGENT by vortex mixing. If small sediment aggregates are visible, they can be disrupted by either (A) brief ultrasound sonication for 15-30 seconds {Recommended}, or (B) returning the centrifuge tubes to the oven/boiling water bath for 15 min. and repeating the combination of heating and vortexing until all large aggregates are dispersed. The suspension is then transferred to the smaller 15 ml centrifuge tubes with a Pasteur pipet. Following the transfer, vortex mix the suspension in the 15 ml centrifuge tube to homogenize the particulates into suspension.
6. The larger 50 ml centrifuge tube is then carefully rinsed twice with 3-4 ml of the diluted MICROSPHERE COUNTING REAGENT and the washes added to the smaller centrifuge tube. The combined suspension in the 15 ml tube is mixed by inversion. Centrifuge for 15 min., and aspirate the supernate to a level slightly above the visible pellet. Briefly centrifuge the tube to sediment any liquid on the centrifuge wall or cap. Proceed to DETERMINATION OF MICROSPHERES SAMPLE VOLUME.

DETERMINATION OF MICROSPHERE SAMPLE VOLUME

Prior to counting of the colored microspheres, the sample volume for each tissue and reference blood sample must be accurately determined and recorded. Following a brief centrifugation to sediment any liquid on the centrifuge wall or cap, three alternative procedures may be used for volume determination.

1. **Volume Determination of 25 μ L - 400 μ L Sample Volumes.** Unknown sample volumes may be determined by the differential weight between the tarred empty centrifuge tube and the same centrifuge tube containing an unknown volume of sample, both weights without the centrifuge cap. All samples are ultimately suspended in MICROSPHERE COUNTING REAGENT having density of 1.005 mg/ml. It is recommended that an analytical balance with a precision of 0.1 mg be used. Following vortex mixing, the samples preparation is ready for counting.

Example:

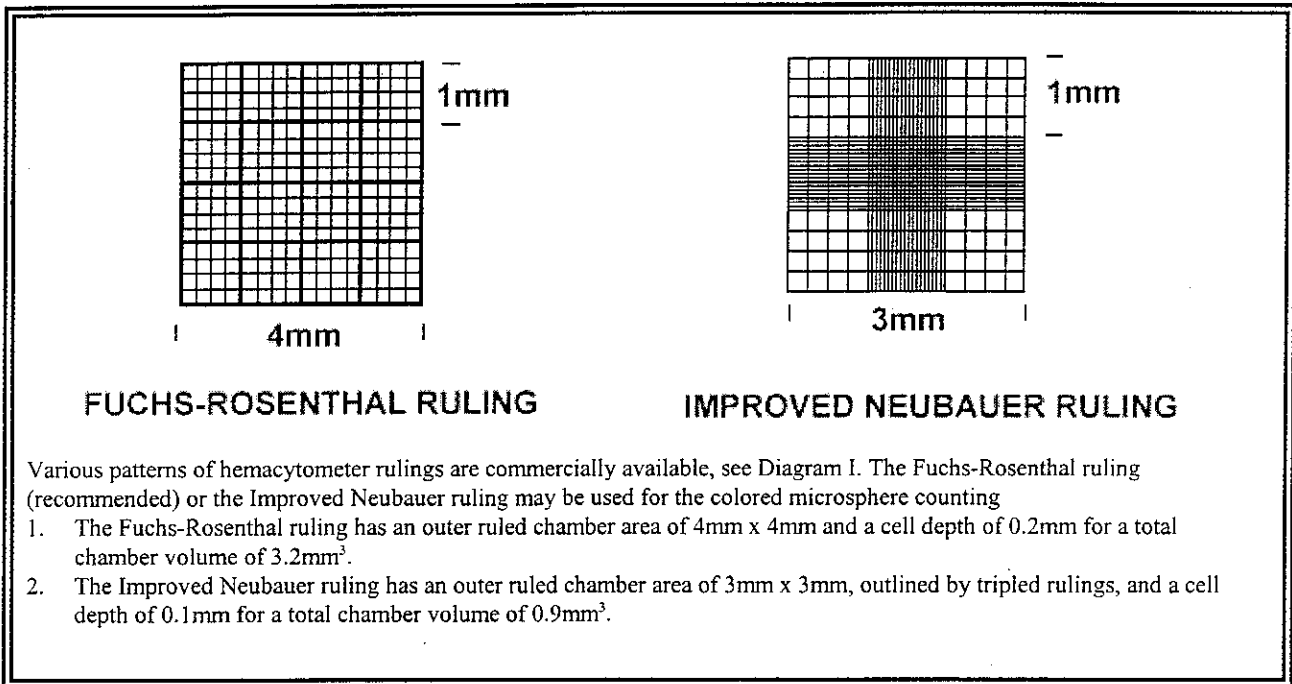
Tarred empty centrifuge tube:	5.5400 g
Centrifuge tube containing unknown sample volume:	<u>5.5830 g</u>
Differential Sample Weight:	0.0430 g
Sample Volume: (0.0430 g / 1.005 g/ml)	0.0428 ml

2. **Volume Determination of 40 μ L - 200 μ L Sample Volumes.** Unknown sample volumes below 200 μ L may be determined using a Continuously Adjustable Digital Pipette (Sugg. Oxford Benchmate 40 to 200 μ L Pipette). The pipette is adjusted to a volume setting of 25 μ L and the microsphere solution sampled at this volume. Without removing the pipette tip from the microsphere solution, the remaining microsphere solution is then brought up into the pipette tip by turning the adjustment knob and increasing the sample volume registered on the digital display. Following the complete withdrawal of the microsphere solution, an air bubble will begin to be drawn into the pipette tip. The air bubble is then carefully expelled from the pipette tip by reversing the direction on the adjustment knob and the total volume for the enclosed microsphere solution is read from the digital display and recorded. Eject the microsphere solution from the pipette tip into the centrifuge tube for vortex mixing before sample counting.
3. **Volume Determination of 200 μ L - 400 μ L Sample Volumes.** Add diluted MICROSPHERE COUNTING REAGENT to each sediment to bring the meniscus to an accurately measured total volume (microspheres plus liquid) corresponding to the 0.20 ml, 0.30 ml, or 0.40 ml markings on the centrifuge tube wall. Following vortex mixing, the sample preparation is ready for counting.

MICROSPHERE COUNTING

The number of colored microspheres in the final tissue and reference blood preparations are determined using a hemacytometer counting slide having a known cell volume. Manual counting of colored microspheres is recommended for researchers performing blood flow measurements on a limited scale. Automated image acquisition and analysis systems are available to speed the counting process.

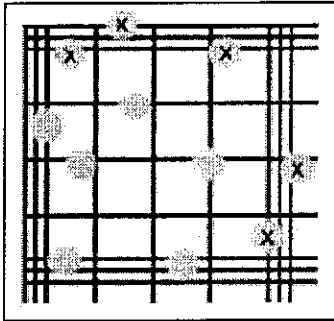
DIAGRAM I



1. The inner rulings on both hemacytometer are used as location aids as one scans the hemacytometer during counting. A microscopic magnification of between 100X and 400X is needed to visualize the microspheres. A 5-key Blood Cell Counter is recommended for tallying the number of colored microspheres as one scans the hemacytometer.
2. Before filling the hemacytometer chambers, the sample preparation must be well mixed by vortexing. With the cover-slip in place, use a Pasteur pipette or other suitable device and transfer a small amount (18µL recommended) of the sample preparation to both chambers of the hemacytometer by carefully touching the edge of the coverslip with the pipette tip and allowing each chamber to fill by capillary action. Care should be taken not to overfill or underfill the chambers, nor to introduce air bubbles. Allow the microspheres to settle in each chamber before counting.
3. Starting in the upper left-hand corner of the outlined hemacytometer chamber, scan the whole hemacytometer chamber in an x-y raster fashion, keeping a separate count for each color of microspheres. According to the perimeter boundary one establishes for a field of view, it is recommended that one follow certain specific rules (see Diagram II) when counting microspheres to prevent repetitive counting of microspheres that straddle the perimeter boundary lines.
4. Errors in microsphere counting may be introduced during sample mixing, by incorrect filling of the chamber or by inaccurate counting. There is an inherent error in counting due to the random distribution of microspheres in the chamber, resulting in a variation of the number of microspheres in chambers of equal size. This variation follows a Poisson Distribution in which the SD = square root of N, where N equals the total sum of microspheres counted. Thus if 200 red microspheres are counted, the SD = square root of 200 = +/-14 microspheres. The coefficient of variation (CV) will be $(14/200 \times 100) = 7\%$. It is apparent that if a smaller number of microspheres are counted, the CV will be greater, e.g. if 100 microspheres are counted it will be 10%. Larger numbers of microspheres may be counted by using the sum of multiple chambers and/or using a chamber with a larger volume, e.g. a Fuchs-Rosenthal hemacytometer.

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DIAGRAM II

<p>Sample field of view through microscope.</p> <p>Count microspheres inside perimeter boundary. Count microspheres touching bottom and left perimeter boundary. Do not count microspheres touching top and right perimeter boundary. Move slide to left to count next square to the right. Microspheres previously on right perimeter will now be on the countable left perimeter boundary.</p>	
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CALCULATION OF BLOOD FLOW

Blood flow values are calculated from the following equation:

$$Q_m = (C_m \times Q_r) / C_r$$

where Q_m is the myocardial blood flow per gram (ml/min/g), C_m is the microsphere count per gram of tissue, Q_r is the withdrawal rate of the reference blood sample (ml/min), and C_r is the microsphere count in the reference blood sample. The example shown is for an injection rate of 5×10^6 red microspheres into the left atrium. The reference blood sample is collected from the femoral artery at a rate of 10.0 ml/min. The tissue biopsy is from the left ventricular myocardium.

Calculation A: Reference Blood Sample

232 red microspheres are counted in one Fuchs-Rosenthal hemacytometer chamber (3.2 mm^3 each). The volume of the final processed suspension is 0.4 ml. The microsphere number in the total reference blood sample (C_r) is calculated as follows:

$$C_r = \frac{232 \text{ microspheres} \times 1000 \text{ mm}^3}{3.2 \text{ mm}^3 \text{ ml}} \times 0.4 \text{ ml suspension}$$

= 29,000 total microspheres in reference blood sample

Calculation B: Myocardial Tissue Sample

258 red microspheres are counted in 4 Fuchs-Rosenthal hemacytometer chambers. The volume of the final processed suspension is 0.4 ml. The initial tissue sample weighed 2.171 gram. The microsphere count per gram of tissue (C_m) is calculated as follows:

$$C_m = \frac{258 \text{ microspheres} \times 1000 \text{ mm}^3 \times 0.4 \text{ ml suspension}}{4 \times 3.2 \text{ mm}^3 \text{ ml} \times 2.171 \text{ g}}$$

= 3,714 microspheres/gram in myocardial tissue sample

Calculation C: Myocardial Blood Flow

$$C_m = 3,714 \text{ microspheres/gram}$$

$$Q_r = 10.0 \text{ ml/min.}$$

$$C_r = 29,000 \text{ microspheres}$$

$$Q_m = (C_m \times Q_r) / C_r$$

$$= 3,714 \times 10.0 / 29,000$$

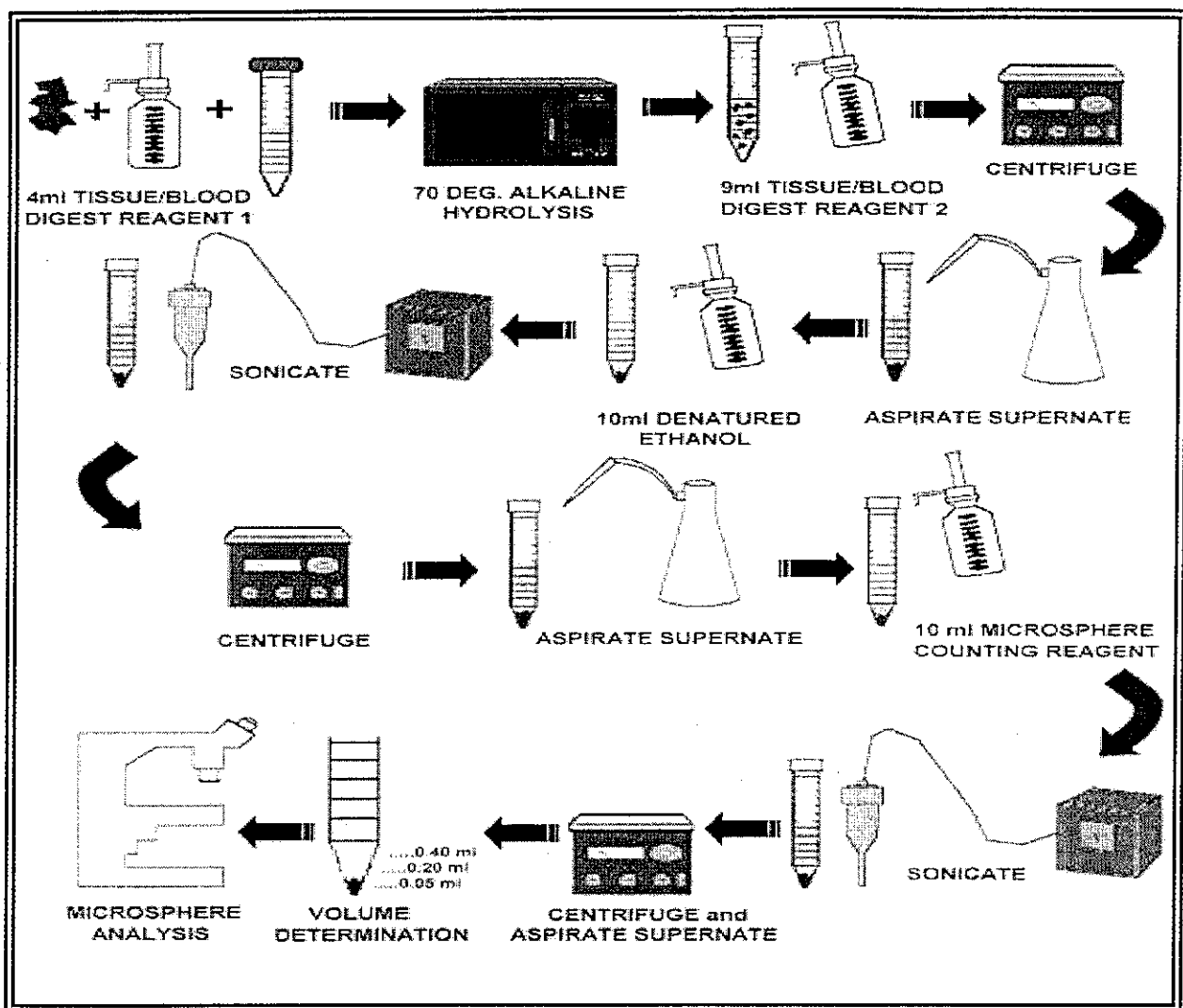
$$= 1.28 \text{ ml/min/g}$$

The above computations may be easily completed using personal computer spreadsheet programs; e.g. Microsoft Excel. Templates compatible with these programs created specifically for use are available from IMT.

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SUPPLEMENTAL PROCEDURE I

RECOVERY OF MICROSPHERES FROM TISSUE SAMPLES COLORED WITH VITAL STAINS (TTC, EVANS BLUE, TRYPAN BLUE)



1. Each whole weighed tissue sample (1-3 gram) is placed in a 15 ml polypropylene centrifuge tube and pushed or centrifuged to the bottom.
2. 4 ml of diluted TISSUE/BLOOD DIGEST REAGENT I (** CAUTION ** Strong Alkaline Solution) is added to each sample. Place the screw caps on the centrifuge tubes and snugly tighten.
3. Either of the following procedures may be employed during the alkaline hydrolysis of the tissue samples.
 - I. Overnight Tissue Hydrolysis: Place the tubes in a general purpose oven {Preferred} or heated water bath at 70°-80°C and allow the tissue to digest overnight. (CAUTION: Cover the water bath with a lid to prevent the evaporation of the heated water.) The following day, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. The tissue samples in each tube should completely homogenize into suspension, with only small particles of fatty white debris visible.
 - II. Same Day Tissue Hydrolysis: Place the tubes in a heated water bath (90°-100°C). The level of the water bath should be even with that of the sample and maintained by periodic additions. Following 15 min. of heating, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. A major portion of the tissue samples in each tube should homogenize into suspension. Return the centrifuge tubes to the boiling water bath for further heating. At periodic intervals (5-10 min.), remove the centrifuge tubes from the water bath and vigorously vortex as described above. This is repeated until all of the tissue is homogenized into suspension and only small

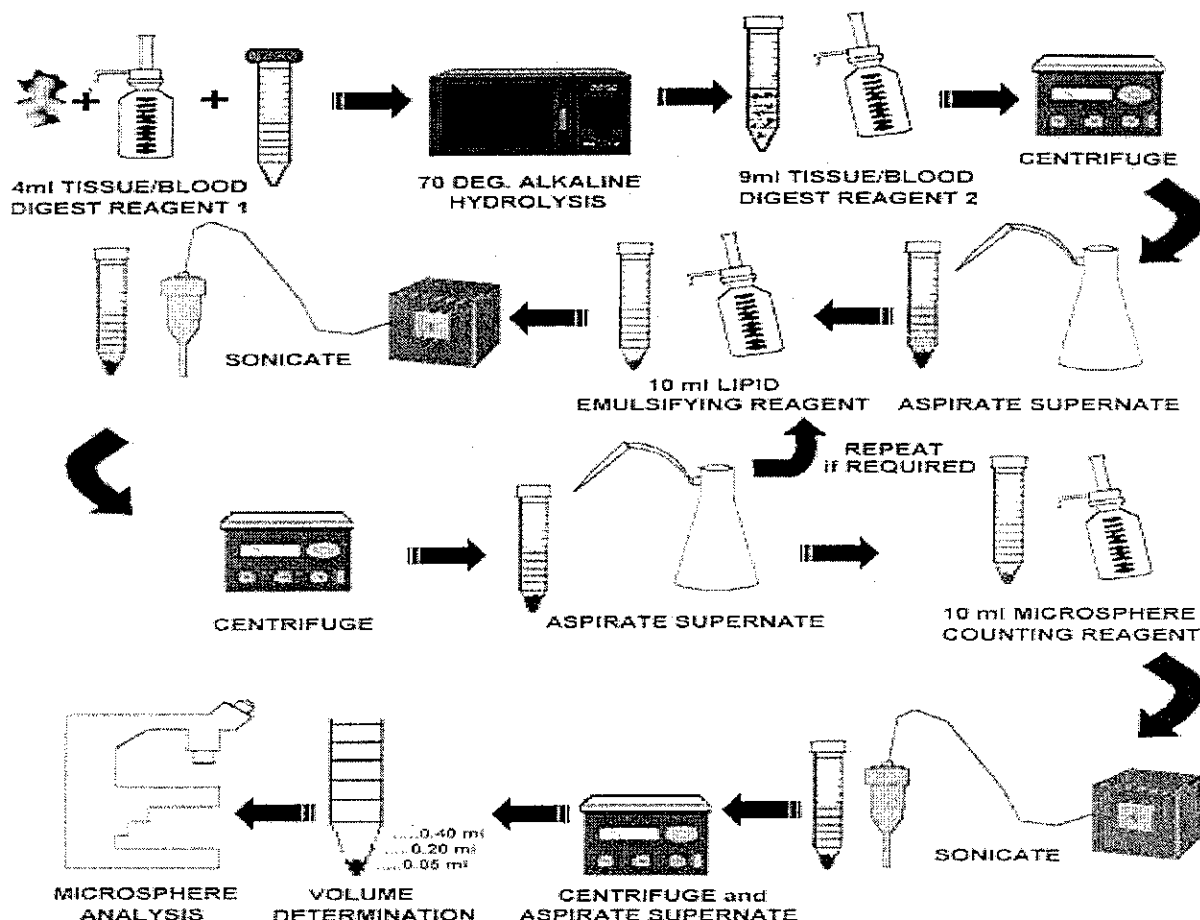
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particles of fatty white debris are visible. Return the centrifuge tubes to the boiling water bath for a final 15-30 min. of heating. Following the alkaline hydrolysis of the tissue samples, proceed immediately to next step, fats and lipids in the sample may gel upon standing.

4. Add diluted TISSUE/BLOOD DIGEST REAGENT II to the sample suspension to bring the total volume to 14-15 ml. Cover with the screw-cap and mix by inversion. Centrifuge the tubes for 30 min. and aspirate the supernate to a level slightly above each pellet.
5. Each greenish-brown sediment is re-suspended in 10 ml of *Denatured ETHANOL* by vortex mixing. Small sediment aggregates should be disrupted by a brief ultrasound sonication for 15-30 seconds.
6. Centrifuge for 15 min., and aspirate the supernate to a level slightly above the visible pellet. If significant amounts of the stain are still visible, repeat the ethanol wash described in step 5.
7. Each brown sediment is re-suspended in 10 ml of diluted MICROSPHERE COUNTING REAGENT by vortex mixing. The small microsphere sediment should be evenly suspended by a brief ultrasound sonication for 15-30 seconds.
8. Centrifuge for 15 min., and aspirate the supernate to a level slightly above the visible pellet.
9. Briefly centrifuge the tube to sediment any liquid on the centrifuge wall or cap. Proceed to DETERMINATION OF MICROSPHERE SAMPLE VOLUME.

SUPPLEMENTAL PROCEDURE II

RECOVERY OF MICROSPHERES FROM TISSUE SAMPLES WITH HIGH LIPID CONTENT (BRAIN, SPINAL CORD)



1. Each whole weighed tissue sample (1-3 gram) is placed in a 15 ml polypropylene centrifuge tube and pushed or centrifuged to the bottom.
2. 4 ml of diluted TISSUE/BLOOD DIGEST REAGENT I (** CAUTION ** Strong Alkaline Solution) is added to each sample. Place the screwcaps on the centrifuge tubes and snugly tighten.
3. Either of the following procedures may be employed during the alkaline hydrolysis of the tissue samples.
 - I. Overnight Tissue Hydrolysis: Place the tubes in a general purpose oven {Preferred} or heated water bath at 70°-80°C and allow the tissue to digest overnight. (CAUTION: Cover the water bath with a lid to prevent the evaporation of the heated water.) The following day, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. The tissue samples in each tube should completely homogenize into suspension, with only small particles of fatty white debris visible.
 - II. Same Day Tissue Hydrolysis: Place the tubes in a heated water bath (90°-100°C). The level of the water bath should be even with that of the sample and maintained by periodic additions. Following 15 min. of heating, remove the centrifuge tubes from the bath and vigorously vortex mix for approximately 15-30 seconds. A major portion of the tissue samples in each tube should homogenize into suspension. Return the centrifuge tubes to the boiling water bath for further heating. At periodic intervals (5-10 min.), remove the centrifuge tubes from the water bath and vigorously vortex as described above. This is repeated until all of the tissue is homogenized into suspension and only small particles of fatty white debris are visible. Return the centrifuge tubes to the boiling water bath for a final 15-30 min. of heating. Following the alkaline hydrolysis of the tissue samples, proceed

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immediately to next step, fats and lipids in the sample may gel upon standing.

4. Add diluted TISSUE/BLOOD DIGEST REAGENT II to the sample suspension to bring the total volume to 14-15 ml. Cover with the screw-cap and mix by inversion. Centrifuge the tubes for 30 min. and aspirate the supernate to a level slightly above each pellet.
5. Each milky white sediment is re-suspended in 10 ml of LIPID EMULSIFYING REAGENT by vortex mixing. Small sediment aggregates should be disrupted by a brief ultrasound sonication for 15-30 seconds.
6. Centrifuge for 15 min., and aspirate the supernate to a level slightly above the visible pellet. If significant amounts of white lipid are still visible, repeat the wash described in step 5.
7. Each brown sediment is re-suspended in 10 ml of diluted MICROSPHERE COUNTING REAGENT by vortex mixing. The small microsphere sediment should be evenly suspended by a brief ultrasound sonication for 15-30 seconds.
8. Centrifuge for 15 min., and aspirate the supernate to a level slightly above the visible pellet.
9. Briefly centrifuge the tube to sediment any liquid on the centrifuge wall or cap. Proceed to DETERMINATION OF MICROSPHERE SAMPLE VOLUME.