DYE-TRAK

Tissue and Blood Processing and Microsphere Recovery

By

FILTRATION

June, 2006
(ver. 0606)

This procedure is an improved version of the original Dye-Trak tissue processing and microsphere recovery procedure. This procedure uses *vacuum filtration* to recover the microspheres from the digested tissue or blood samples. An alternate method using *sedimentation* is described in another procedure. Each of these procedures has it's advantage and disadvantages. We recommend that new users read through both procedures and select the method that best suits their needs.

This **filtration** procedure can be used for either the 'original' ***5-color* Dye-Trak** microspheres or ***7-color* Dye-Trak VII+** microspheres. The significant difference between the two families is that the original Dye-Trak microsphere family uses DMF as the solvent to extract dye, while Dye-Trak VII+ uses *acidified* Cellosolve Acetate as the solvent. In the case of Dye-Trak VII+ that the Cellosolve Acetate must be acidified using the in the recipe in the Reagent Section of this procedure.

Original Dye-Trak Colors:

White (370nm), **Yellow** (448nm), **Eosin** (535nm), **Violet** (594nm), **Blue** (672nm)

Recommended microspheres combinations for various numbers of experimental colors using original Dye-Trak 5-color family are listed below. These color combinations are selected to minimize spill-over between colors.

Color Selection Table for *original* 5-color Dye-Trak family

Number of Colors Used	Colors	Solvent	Control Color
1	**Yellow**	**DMF**	**Blue**
2	**Yellow and Eosin**	**DMF**	**Blue**
3	**Yellow, Eosin, and White***	**DMF**	**Blue**
4	**Yellow, Eosin, White* and Violet**	**DMF**	**Blue**
5	**Yellow, Eosin, White*, Violet and Blue**	**DMF**	**none**

**We recommend that White be read at 390nm, rather than it's peak absorbance to to minimize biologic UV baseline background noise.*

.Dye-Trak VII+ Colors:

Lemon (390), **Yellow** (440nm), **Orange** (495nm), **Tangerine** (525nm),
Persimmon (545nm), **Berry** (5940nm), **Blue** (672nm)

Recommended microspheres combinations for various numbers of experimental colors using **Dye-Trak VII**+ family are listed below. These color combinations are selected to minimize spill-over between colors.

Color Selection Table for Dye-Trak VII+ family

Number of Colors Used	Colors	Solvent	Control Color
1	**Yellow**	***acidified*** **Cellosolve Acetate**	**Blue**
2	**Yellow and Persimmon**	***acidified*** **Cellosolve Acetate**	**Blue**
3	**Yellow, Persimmon , and Orange**	***acidified*** **Cellosolve Acetate**	**Blue**
4	**Yellow, Persimmon, Orange, and Lemon***	***acidified*** **Cellosolve Acetate**	**Blue**
5	**Yellow, Persimmon, Orange, Lemon* and Tangerine**	***acidified*** **Cellosolve Acetate**	**Blue**
6	**Yellow, Persimmon, Orange, Lemon*, Tangerine and Berry**	***acidified*** **Cellosolve Acetate**	**Blue**
7	**Yellow, Persimmon, Orange, Lemon*, Tangerine, Berry, and Blue**	***acidified*** **Cellosolve Acetate**	**none**

White can be substituted for Lemon. We recommend that White be read at 390nm, rather than 370nm in order to minimize background.

1.0 Sample Preparation

Process Control:

Triton Technology feels that using a **Process Control** step is an ***essential requirement*** to proper microsphere recovery. Process Control is accomplished by adding a known number of Blue microspheres to each tissue or blood sample prior to the alkaline tissue digestion. The Process Control spheres are then used as an internal check for any loss of microspheres during tissue digestion and sample processing. This Process Control step determines the efficiency of microsphere recovery and this data can be used to correct the measured results for each tissue or blood sample.

Process Control *microspheres costs less a few cents per tissue sample!*

If the amount of Process Control dye detected in a recovered sample is less than was initially added to the tube prior to digestion, then some proportion of spheres in the sample were lost during processing. This 'loss' percentage data can be used to correct the resulting Dye-Trak measurements for the loss factor. The free Excel blood flow calculation macros that Triton supplies will correct any detected loss of microspheres in an individual sample.

Process Control is accomplished by using a repeating pipette to add fixed number of microspheres of Blue microspheres (typically10,000 **Blue** microspheres), to each sample tube prior to adding the KOH. Blue are used for this purpose because they are at the long-wavelength end of the spectrum and other colors do not spill-over into it's peak-absorbance wavelength. The instructions for preparing a Process Control solution can be found in the **Reagents Recipes** section of this procedure.

Recovery Standard:

In addition to adding Process Control spheres to each tissue and blood sample prior to processing, several new empty tubes should also be prepared with Process Control spheres alone (no tissue). Add 100µL of the Process Control spheres to each of three to five empty control tubes. Set these tubes aside as they will not go through any processing and digestion steps. Later, these control tubes will be analyzed and these Process Control AU readings will serve as the **100% recovery** standard to which all the Process Controls in the sample tubes will be compared. The Triton Technology Excel spreadsheets use this information to determine the 100% recovery value for the Process Control microspheres in the sample analysis steps.

1.1 Tissue Sample

1. Harvest tissue samples from the tissue beds of interest. Tissue samples may be stored in capped tubes at room temperature for very short-term storage or at 0-4° C for longer term storage. If tissue samples are kept un-refrigerated for long periods of time, consider working under a fume hood when the tubes are uncapped.

   Alternatively, the tissue samples may be fixed with formalin. *If tissue-fixing agents other than formalin are to be used, the fixed tissues should be tested using the complete tissue processing procedure in order to determine if there are any digestion or recovery problems. Spectrophotometric analysis must also be evaluated to confirm dye absorbance profile and intensity stability before using alternate tissue fixing agent.*

2. Tissue samples must be processed in **new** disposable polypropylene centrifuge tubes appropriate to the tissue sample size. Experience has shown that there can be a significant loss of spheres during tissue processing when using used centrifuge tubes.

   Screw-top Glass centrifuge tubes can also be used for processing of the tissue and blood samples

   Polystyrene centrifuge tubes should *not* be used as polystyrene tubes are quite brittle and might crack during the processing procedures.

3. **Sample Preparation:**

   Small tissue samples can be processed in 15 or 50ml centrifuge tubes. We have found that using 50ml tubes, even for small tissue samples, results in better digestion and cleaner samples. The advantage of this is that it is less likely that the filters will clog during microsphere recovery and dye analysis.

   Pre-weigh each empty centrifuge tube and 'tare' out the tube weight. . Weigh the centrifuge tubes with their caps removed. . Each tube should be marked or labeled with an identification number or name written on the outside surface using a marker pen with permanent ink. As the tissue samples are harvested they should be placed in centrifuge tubes, identified with a code. Push the tissue sample to the bottom of it's tube with a rod (or briefly centrifuge each sample). The centrifuge tube is then re-weighed to determine the 'wet-weight' of each tissue sample. The 'wet weight' of each sample should then be recorded along with the **Sample Identification** information. This information will be used later for volume blood flow calculations for each sample (ml/minute/gram).

   A. **Small Tissue Sample** (*3 grams or less):*
   Each small tissue sample is placed in a pre-weighed ('tared') 15 or 50ml polypropylene centrifuge tube. Weigh each sample tube, record the weight of the tissue sample and record the associated tube identification.

   B. Larger Tissue Samples *(3-10 grams):*
   Each large tissue sample is placed in a pre-weighed ('tared') 50mL polypropylene centrifuge tube. Weigh each sample tube, record the weight weight of the tissue sample and record the associated tube identification.

4. **Process Control**
   Use a repeating pipette to add 10,000 Blue Process Control microspheres (100uL) from a constantly-stirred beaker of Process Control solution to each Tissue Sample tube prior to the following processing steps.

5. **Alkaline Digestion Reagent (4M ADR)**:

   *The use of nitrile or latex gloves are strongly recommended for all the sample processing steps, particularly those steps involving the **Alkaline Digestion Reagent**. The KOH digestion reagent is very caustic and will burn exposed skin. Eye protection should also be used.*

   It is important *not* to use a more concentrated KOH solution than 1 Molar when using the *sedimentation* process as the increased density of the solution will make it impossible to centrifuge the microspheres to the bottom of the tubes. Reagent preparation instructions can be found in the Reagent Section of this procedure
   .
   A. **15mL Sample Tube** (a sample size of less than 3grams)**:**
   When using a 15mL centrifuge tube, add 12mL of ***4 Molar Alkaline Digestion Reagent*** **(4M ADR)**. Place the screw cap on each tube and tighten snugly. IMPORTANT: Do not use more than **10mL** of **1molar KOH** in the 15mL tubes.

   B. **50mL Sample Tube** (any size sample):
   When using a 50mL centrifuge tube, add 40mL of ***4 Molar Alkaline Digestion Reagent*** **(4M ADR)**. Place the screw cap on each tube and tighten snugly. IMPORTANT: Do not use more than **30mL** of **4 molar KOH** in the 15mL tubes.

6. Next go **to Section 1.3** of the Procedure, **Overnight Alkaline Hydrolysis.**

1.2 Blood Sample

All Reference blood samples should be treated with an anti-coagulant as soon as the blood is withdrawn. For example, add 1.5mg of EDTA for each milliliter of blood in the Reference Blood Sample. This should be mixed by repeated inversion to prevent blood coagulation prior to processing. Coagulated blood is difficult to digest. Heparin or Citrate may also be used as an anti-coagulant.

1. All reference Blood Samples should be processed in **new 50mL** tubes. Reference Blood Samples larger than **10mL** must be split and processed in multiple 50mL centrifuge tubes. N**ew 50mL** disposable polypropylene centrifuge tubes should be used to minimize the potential for microsphere loss during processing.

   Screw-top Glass centrifuge tubes can also be used for processing of the tissue nd blood samples

   Polystyrene centrifuge tubes should *not* be used, as polystyrene tubes are quite brittle and may crack during the repeated centrifuge steps.

2. **Process Control**
   Use a repeating pipette to add 10,000 Blue Process Control microspheres (100ul) from a constantly-stirred beaker Process Control solution to each Blood Sample tube prior to the following processing steps.

3. **Alkaline Diegestion Ragent (16M KOH)**
   Each 10mL or less blood sample tube should be diluted up the 30mL level with 2% Tween-80 and vortex mixed. Add 10mL **16M KOH** and votex mix.

4. Next go **to Section 1.3** of the Procedure, **Overnight Alkaline Hydrolysis**

1.3 Overnight Alkaline Hydrolysis

*The use of nitrile or latex gloves are strongly recommended for all the sample processing steps, particularly those steps involving the **Alkaline Digestion Reagent**. The KOH digestion reagent is very caustic and will burn exposed skin. Eye protection should also be used*

It is assumed that a number of tissue / blood samples will be processed at the same time. The number of tubes that can be held in the centrifuge will limit the number of tubes that are process at one time.

It is recommended that *sonication* be used in each of the following steps to break up the pellet and re-suspend any remaining undigested tissue. The break-up of tissue pieces can be accelerated with a brief 'sonication' using an ultrasonic tissue hemoginizer (see Hints and Notes). It is very helpful to use the sonicator to thoroughly break-up the microsphere pellet at the bottom of the tube. While sonicating, move the probe-tip repeatedly from the bottom to the top to the bottom of the centrifuge tube, thoroughly sonicating all the material into a homogeneous suspension. After each tube is 'sonicated', use a wash bottle filled with distilled water to rinse the sonicator probe-tip back inot the sample tube so that no microspheres are lost.

If sonication unavailable, aggressive vortexing will be required to re-suspend the tissue and microsphere pellet in the following digestion steps.

1. Place the tubes from the tissue and blood preparation steps in a temperature-controlled laboratory oven set to a maximum 60°C and allow the tissue/blood samples to digest overnight. After overnight digestion, remove the sample tubes from the oven, briefly loosen the screw caps to vent gas pressure, then snugly re-tighten the screw caps, then thoroughly vortex-mix the contents of the tube for approximately 15-30 seconds. The tissue/blood samples in each tube should completely homogenize into suspension, (only small particles of fatty white debris should be visible in the sample tubes). Return the samples to the oven for an additional hour of digestion.

2. After an additional hour of digestion, repeat the sonication or vortex-mix steps described above. Visually inspect the samples again. If undigested pieces of tissue remain visible, continue digestion at 60°C throughout the day with intermittent vortex mixing. Keep the samples warm in the oven until you are ready to filter the digestate. Warm samples will filter much easier than samples that are at room temperature. If or any reason sample are allowed to cool, they should be re0warmed in the 60°C oven prior to the microsphere recovery step.

3. Next, go to **Section 1.4** of the Procedure, **Microsphere Recovery**

1.4 Microsphere Recovery, Dye Extraction and Analysis:

1. Assemble the filtration apparatus using a 25mm diameter 10 μm pore size filter (Triton Technology Part #31079) between the upper graduated cylinder and the filter screen per the manufacturer's directions. Take care to insure that the filter is centered in the apparatus to prevent any unnecessary loss of microspheres during filtering. Clamp the graduated cylinder to the filter holder carefully so as not to disturb the filter alignment.. Attach the vacuum connection, turn the vacuum source on. Pre-rinse the inside of the filter apparatus and the filter with a small amount of ETOH. Turn the vacuum source off.

2. Remove a tissue or blood sample from the 60°C oven, vortex mix the sample and pour the contents on into the graduated cylinder. After the sample is in the cylinder above the filter, turn-on the vacuum source. When the contents of the cylinder have completely passed through the filter, turn the vacuum source off. Adding the sample filtrate while vacuum is not operational results in a more even distribution of the microspheres and debris on the filter membrane when the vacuum is applied.

   It is helpful to have a foot-switch control for the vacuum source.

3. Rinse the sample tube with approx. 10mL of 60°C distilled water from a beaker kept warm on a hot plate, vortex mix and pour the contents into the graduated cylinder. This is done to insure the full recovery of microspheres that might remain on the walls of the tube. Hot water is used to keep any fats in solution and prevent the filter from clogging. Turn the vacuum source ON to complete the filtering. When the filter is dry, rinse the walls of the filtration graduated cylinder with more heated distilled water. Turn off the vacuum source when the complete contents have passed through the filter and the filter is dry.

4. Rinse the sample centrifuge tube with ETOH from a wash bottle, vortex mix and pour the contents into the graduated cylinder. An ETOH rinse will collapse water-based bubbles on the walls of the centrifuge tube and recover any remaining microspheres. Turn on the vacuum source, when the filter is dry, rinse the walls of the graduated cylinder with distilled water. Turn off the vacuum source when the complete contents of the cylinder have passed through the filter.

5. Add distilled water to the graduated cylinder to the marked 10mL level. Initiate vacuum filtration, when the filter is dry, rinse the walls of the graduated cylinder with distilled water. Turn off the vacuum when the complete contents have passed through the filter. Repeat this step a second time. At this point the presence of residual KOH has been minimized.

6. Add ETOH to the graduated cylinder to the marked 10mL level. Turn on the vacuum filtration, when the filter is dry, rinse the walls of the graduated cylinder with ETOH from a wash bottle.When the filter is dry once again, with the vacuum source still on, remove the clamp, and the upper graduated cylinder portion of the filter apparatus.

7. Continue filtration vacuum until the filter has become completely air-dried, then turn off the vacuum.

8. Carefully remove the filter from the filter apparatus support screen with tweezers or a similar tool. Handle the filter only by the edge where there will be no microspheres. Keep the filter level with the 'microsphere side' upwards. With care, partially form the filter into a U shape and place the filter into the upper portion of a 15mL polypropylene centrifuge tube while holding the centrifuge tube horizontal. Always keep the 'microsphere side' of the filter always facing upward. The filter should now be resting on the bottom wall of the horizontal tube. With care not to disturb microspheres resting on the upper surface of the filter, push the filter to the bottom of the centrifuge tube using a spatula or similar tool.

9. Hold the centrifuge tube vertical and add 300 μL of the appropriate solvent, for a 200μL cuvette measurement. Use **DMF** for Original 5-color Dye-Trak. Use ONLY ***acidified*** **Cellosolve Acetate** solvent for **Dye-Trak VII+**.

   Failure to use the ***acidified Cellosovlve Acetate*** *with Dye-Trak VII+ will cause variable results !*

10 Vortex mix. Insure that the solvent completely covers the filter when vortex mixing. If necessary, use the spatula to push the filter further down into the solvent allowing the solvent to completely solvate all portions of the filter. Allow the tubes to stand for approximately 1 hour, with vortex mixing every 15 minutes. Remove the filter from the tube using a spatula and discard the filter.

11. Centrifuge each tube for 10 minutes at 1500g. *It is recommended that any centrifuge brake be disabled during centrifuge deceleration period. Braking can tend to resuspend small particles that add noise to the absorbance measurements at the UV end of the spectrum.*

12. Analyze each sample by absorbance spectrophotometry using an appropriate volume microcuvette (100 to 200 μl). Carefully extract the dye solvent sample from the sample tube using an adjustable pipette set for 200 μL. It is important to extract the analysis sample from well above the sediment so as not to disturb the microsphere/debris pellet on the bottom of the tube. Add the sample to the cuvette and read the sample at the appropriate wavelengths.

    It may be necessary to remove some dye samples from the cuvette and dilute them with solvent in order to keep the absorbance readings in the linear region. Absorbance readings should be made below 1.5AU for the highest absorbance peak in order to keep the measurements in the linear region of dye analysis. It is very important to record any dilutions so that this information is used for the final data analysis.

13. The spectrographic data for each tissue and blood sample should be recorded. Most contemporary spectrophotometers will generate a ASCII (or similar) data file for file storage. It is not necessary to do a complete scan, it is only necessary to read the samples at the specific wavelength for each color of microsphere used. Triton Technology supplies free Excel software macros for the blood flow analysis that sequentially opens and imports data from archived ASCII stored analysis file. These macros will import the data files from most spectrophotometers. Using the user-supplied tissue weights and Reference Blood information, the Excel file will calculate blood flow for each sample. Contact Triton Technology for information on the free software Excel compatible macros (triton2@cts.com).

1.5 HINTS AND NOTES:

1. Tissue from the gastrointestinal (GI) track, even when less than 3 grams, should be processed in the larger 50mL centrifuge tubes due to the presence of an unknown biologic gelling component. Following alkaline digestion, re-suspend the GI samples in ***10% Triton X-100 Reagent***. Following sonication, each sample may require heating to 50°C to emulsify solid fatty particulates or to liquefy the gel. Periodic vortexing will assist the re-suspension process. Centrifuge each tube while warm. Repeat these steps as required to achieve a clear supernate solution.

2. The Ultrasonic Homogonizer ('Sonicator') is a *crucial and necessary piece of equipment* for the processing procedure. It is used at many phases of the tissue digestion and the subsequent microsphere recovery steps. The sonication will accelerate and complete the mechanical break-up of tissue aggregates by sending shock waves throughout the tissue suspension. It is used following each centrifugation step to re-suspend microsphere/tissue debris pellets. The Ultrasonic probe tip should be narrow enough to reach the bottom of a 15mL tube (approx. 2mm diameter).

   An ultrasonic cleaning bath will not work as a substitute; the sound waves do not effectively penetrate the plastic walls.

3. The wearing of nitrile or latex gloves are recommended for all sample processing steps, particularly steps involving the ***Alkaline Digestion Reagent*** as it is very caustic. Eye protection should be used when working with KOH solutions.

1.6 Processing Equipment, Chemicals and Reagents:

1.6a Equipment:

UV/VIS Spectrophotometer (should be capable of generating ASCII data files to computer or floppy disk)

100uL to 200ul UV/VIS spectrophotometer microcuvette for spectrophotometer

Ring-Stand with clamps, bars, etc.

200ul Adjustable Pipetter with disposable tips

Bench-Top Centrifuge, with buckets and holders compatible with 50mL and 15mL centrifuge tubes.

Vacuum Aspirator set-up with bubble trap and waste collection reservoir.

Vacuum source, preferably connected to a foot control switch.

Pasteur pipettes with rubber suction bulbs

Filter Apparatus (Fisher

Triton Technology Inc 25 mm diameter filter membranes, 10μm pore size (#31079)

50mL Conical Polypropylene Screw-top Centrifuge Tubes (FALCON 35-2098 or equiv.)
Note: DO NOT use polystyrene centrifuge tubes, this plastic is too brittle!

15mL Conical Polypropylene Screw-top Centrifuge Tubes (FALCON 35-2096 or equiv.)
Note: DO NOT use polystyrene centrifuge tubes, this plastic is too brittle!

The use of an ultrasonic tissue hemogenizer is strongly recommended:
The sonicator greatly speeds up the tissue processing and improves the completeness of the tissue digestion.

Ultrasonic Processor with Probe 6mm ProbeTip, 70-Watt (COLE-PARMER P-04714-00 or equiv.)

3 mm Titanium Ultrasound Probe Tip (COLE-PARMER P-04712-12 or equiv.)

Ultrasonic Processor Footswitch (recommended) (COLE-PARMER P-04712-05 or equiv.)

Tissue & Blood Processing equipment, chemicals and reagents (continued):

1.6b Reagents:

Below is a list of the reagents which are used in the tissue digestion and microsphere recovery procedures. Item numbers from the Sigma-Aldrich catalog are included for reference (www.Sigma-Aldrich.com). All reagents should be A.C.S. grade or better.

ETOH - Denatured Ethyl Alcohol, 1 liter ..... **Sigma-Aldrich** 27,074-1

Tween 80, 25mL ..... **Sigma-Aldrich** 27,436-4

Triton X-100, 1 Liter ..... **Sigma-Aldrich** 27,074-1

Potassium Hydroxide, pellets, (FW 56.11, 1kg) .... **Sigma-Aldrich** 22,147-3

Hydrochloric Acid, 37%, 500mL ..... **Sigma-Aldrich** 25,814-8

Sodium Azide*, (FW 65.01), 5 gram ..... **Sigma-Aldrich** 19,993-1

Thimerosal, (FW 404.8), 1 gram ..... **Sigma-Aldrich** E3,525-1
(Ethylmercurithiosalicylic Acid, sodium salt)

Sodium Chloride, (FW 58.44), 100 gram ..... **Sigma-Aldrich** 22,351-4

DMF** *N*,*N*-Dimethylformide, *spectrographic grade*, 1 liter ... **Sigma-Aldrich** 15,481-4

or

Cellosolve Acetate** 2-Ethoxyethyl Acetate, 1 liter ..... **Sigma-Aldrich** 10,996-7

* Users may substitute 0.1 gram of Thimerosal per liter instead of Sodium Azide in the Triton X-100 solutions. Sodium Azide and Thimeosal are used a bacteriostats in solutions that will be stored for some time.

** DMF is used with the original 5-color Dye-Trak microsphres and acidified Cellosolve Acetate is used with the Dye-Trak VII+ family

1.7 Processing Reagents - Recipes:

A Material Safety Sheet (MSDS) should be obtained for any reagent being used and appropriate handling steps should be observed All prepared reagents should be stored at room temperature.

4M KOH Alkaline Tissue Digestion Reagent:
Add 1000mL of distilled water to a 1-liter glass beaker. Place the beaker on a magnetic-stirring hot plate. Place a magnetic stirring bar into the water and begin stirring at a fairly rapid rate. Add 224.4 gram of Potassium Hydroxide pellets to the water and stir until the solution becomes clear. Turn off the heat and cool to room temperature with continuous stirring. Store the solution in plastic bottles identified as **"4M KOH Alkaline Digestion Reagent (4M KOH").** *Caution: This is a very caustic solution. Wear Latex gloves and handle with extreme care !*

16M KOH Alkaline Blood Digestion Reagent:
Add 1000mL of distilled water to a 1-liter glass beaker. Place the beaker on a magnetic-stirring hot plate. Place a magnetic stirring bar into the water and begin stirring at a fairly rapid rate. Add 897.8gram of Potassium Hydroxide pellets to the water and stir until the solution becomes clear. Turn off the heat and cool to room temperature with continuous stirring. Store the solution in plastic bottles identified as **"16M KOH Alkaline Blood Digestion Reagent (16M KOH").** *Caution: This is a very caustic solution. Wear Latex gloves and handle with extreme care!*

Acidified Cellosolve Acetate:
Prepare a stock solution of *acidified cellosolve acetate* by adding 10µL hydrochloric acid (HCl, 37%/10N) to 100mL of Cellosolve Acetate**.** Store in a screw top plastic bottle identified as ""**Acidified Cellosolve Acetate (ACA)"**

0.05% Tween® 80 - Saline Solution (Microsphere Carrier Solution):
Add 0.50mL 10% **Tween®** 80 solution to 99.5mL sterile saline solution and mix by stirring. Make the reagent as needed for use as an injectate solution. Store in plastic bottles identified as **"Saline plus 0.05%Tween 80 (MCS)".**

Process Control (stock solution):
Make up a solution with a concentration of 100,000 Blue spheres per mL using the ***Microsphere Carrier Solution*** **(MCS)** to dilute the factory concentration of 3 million microspheres per mL. Use a 100µL 'repeating-pipette' to add **Process Control** spheres to each tissue/reference processing tube prior to hydrolysis. The **Process Control** solution should be continuously stirred while filling the 100µL repeating pipette to insure that each **Process Control** bolus contains the same number of spheres.