

# DYE-TRAK

## Tissue and Blood Processing and Microsphere Recovery

By

## SEDIMENTATION

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

This sedimentation procedure can be used for either the 'original' **5-color Dye-Trak** family of microspheres or the **7-color Dye-Trak VII+** family of colored 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. It is important that the correct solvent be used. In the case of Dye-Trak VII+ , it is very important that the acidified Cellosolve Acetate is prepared 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 to Use	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 of 370nm in order to minimize biologic UV background noise.

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### Dye-Trak VII+ Colors:

**Lemon** (390), **Yellow** (440nm), **Orange** (495nm), **Tangerine** (525nm), **Persimmon** (545nm), **Berry** (594nm), **Blue** (672nm)

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

### Color Selection Table for Dye-Trak VII+ family

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

\*White can be substituted for Lemon. We recommend that White be read at 390nm, rather than it's peak absorbance of 370nm in order to minimize biologic UV background noise.

### 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 (typically 10,000 **Blue** spheres, to each sample tube prior to adding the KOH. Blue microspheres 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 100 $\mu$ L 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 short-term storage or at 0-4°C for longer-term storage. If tissue samples are kept un-refrigerated for long periods of time, you should 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. Our experience has shown that there can be a microsphere loss during tissue processing when 'previously used' centrifuge tubes are used.

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

Polystyrene centrifuge tubes should *never* be used, as polystyrene tubes are quite brittle and may crack during the repeated centrifuge steps.

3. **Sample Preparation:**

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') 15mL 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.

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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 (1M 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 6mL of **1 Molar Alkaline Digestion Reagent (1M ADR)**. Place the screw cap on each tube and tighten snugly. **IMPORTANT:** Do not use more than **6mL** of **1molar KOH** in the 15mL tubes

**B. 50mL Sample Tube** (a sample of greater than 3grams):

When using a 50mL centrifuge tube, add 20mL of **1Molar Alkaline Digestion Reagent (1M ADR)**. **IMPORTANT:** Do not use more than **20mL** of the **1 molar KOH** in the 50mL tubes.

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

### 1.2 Blood Sample

The Blood Hemolysis Reagent (BHR) should be added to the Reference Blood Samples as soon as they are withdrawn. If BHR cannot be added to the blood as it is collected, then the blood should be anti-coagulated with EDTA (1.5mg/ml of blood) as soon as it is withdrawn. Mix by repeated inversion to prevent blood coagulation prior to the time that the BHR is added for processing. Heparin or Citrate may also be used as anti-coagulants. It is extremely important to prevent the blood from coagulating, as coagulated blood is very difficult to digest.

1. All reference Blood Samples should be processed in **new 50mL** tubes. Reference Blood Samples larger than **20mL** must be split and processed in multiple 50mL centrifuge tubes. **New 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 and blood samples

Polystyrene centrifuge tubes should *never* 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 of Process Control solution to each Reference Blood Sample tube prior to the following processing steps.

3. If the **Blood Hemolysis Reagent (BHR)** has not been added to the anti-coagulated blood it should be done at this time. Fill each Blood Sample tube (containing 20ml or less of anti-coagulated blood) up to the top mark (50ml) and mix by inversion.. It is important to correctly identify and keep track of blood samples that have been split into multiple 50ml tubes for processing, as this data will be recombined for the blood flow calculations.

4. Centrifuge the tubes for 15 min. at 1,500g and aspirate the supernate to a level safely above visible pellet. The pellet will not be easy to see, so when in doubt, aspirate *no lower than the 10mL volume mark*.

5. **Alkaline Digestion Reagent (1M 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 KOH solution more concentrated than 1 molar when using the **Sedimentation** process. The increased density of the solution will make it impossible to centrifuge the microspheres to the bottom of the tube for recovery. The 1 molar KOH reagent preparation instructions can be found in the Reagent Recipes section of this procedure.

Add 20mL of **Alkaline Digestion Reagent (ADR)** to each Reference Blood centrifuge tube (50mL). Direction for the preparation of **ADR** can be found in the Reagents Recipes section of this procedure. It is very important to place a screw cap on each tube and tighten snugly.

**Do not** add more than **20mL** of **ADR** in any of the 50mL Blood Sample tubes !

6. 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 strongly recommended that an ultrasonic 'sonicator' be used in each of the following steps to break-up the tissue pellet and re-suspend any remaining undigested tissue. The break-up of tissue pieces can be accelerated with a brief 'sonication' using the ultrasonic tissue homogenizer (see Hints and Notes) 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 in to 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
3. Remove all tubes from the oven and fill each tube to the top mark with 50°C distilled water. *Do not overfill the tubes!* Cap the tubes and mix the contents by repeated inversion.
4. Centrifuge the sample tubes for 15 min. at 1,500g (typically 2500 rpm) and aspirate the brown-green supernatate to a level *safely* above the visible pellet in each tube (0.5-1 cm above the bottom of the tube).
5. Re-suspend the tissue/blood pellet with the **10% Triton X-100 Reagent** Use 12mL reagent in the 15mL tubes or 40mL reagent in the 50mL tubes.

The following steps will require sonication or vortexing to help the digestion process. Don't over-fill the tubes as it will make vortexing difficult or cause the liquid to overflow when the sonicator tube is inserted in the tube. This will cause microspheres to be lost!

When processing **brain** or **spinal cord** tissues, substitute **15% Triton X-100 Reagent** and repeat Steps 5 and 6 twice (re-suspension, sonication or vortexing, and centrifugation).

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6. Centrifuge the tubes for 5 min. at 1,500g and again aspirate the supernate in each sample tube to a level *safely* above each visible pellet.
7. Re-suspend the tissue/blood pellet with the ***Acidified Ethanol Reagent (AE)*** Use 12mL reagent in the 15mL tubes or 40mL reagent in the 50mL tubes. Sonicate or vortex mix the contents of the tube to break-up the pellet. Use a wash bottle filled with ETOH to wash sonicator probe-tip back into sample tube after each sonication step.
8. Centrifuge the tubes for 5 min. at 1,500g and aspirate the supernate in each sample tube to a level safely above each visible pellet.
9. Re-suspend the tissue/blood pellet with the **ETOH**. Use 12mL reagent in the 15mL tubes or 40mL reagent in the 50mL tubes. Sonicate or vortex mix the contents of the tube to break-up the pellet. Use a wash bottle filled with ETOH to wash sonicator probe-tip back into sample tube after each sonication step.
10. Centrifuge the tubes for 5 min. at 1,500g and again aspirate the supernate in each sample tube to a level safely above each visible pellet.
11. Next, go to **Section 1.4** of the Procedure, **Microsphere Recovery**

### 1.4 Microsphere Recovery and Dye Analysis

Each day when a dye analysis session is to be done, the spectrophotometer should have a baseline calibration. The baseline calibration should be performed by doing a 'step-wise' absorbance scan, with only solvent in the cuvette. The scan should be across the entire wavelength spectrum of interest, typically 350 to 700nm. Refer to the manual for your spectrophotometer to understand how to do a baseline calibration scan.

There are two alternate ways to recover the dye from the microspheres and analyze the recovered colored dye with the spectrophotometer:

*Important: Which ever option is used, good lab technique is always important. The cuvettes should always be clean inside and out, with no finger- prints on the outside surface. The cuvette should be flushed out with ETOH and completely dried between dye analysis steps to prevent any dye carry-over between samples.*

*Important: Use DMF for original 5-color Dye-Trak or acidified Cellosolve Acetate for 7-color Dye-Trak VII+ microspheres.*

#### Evaporation Recovery of Microspheres and Dye

1. Allow the microsphere sample pellet and small amount of ETOH from Section 1.3-Step 10 to evaporate dry overnight at room temperature. Drying can be done faster in a temperature-controlled oven at a maximum temperature of 50°C. *Caution: At temperatures higher than 50°C, the plastic centrifuge tube may possibly absorb some of the eluted dye.*

If a tissue or blood sample has been split into multiple tubes, now is the time to recombine them. Using ETOH as a medium, suspend the spheres, vortex mix and transfer all the parts of one tissue or blood into one sample tube. Thoroughly rinse each of the originating tubes with ETOH and transfer into the remaining sample tube. Centrifuge the sample tube to force the microspheres to the bottom of the tube, aspirate off the excess ETOH and proceed as with a single tube.

2. Add 150 to 250µL of the appropriate solvent to the dried microspheres in each sample tube and vortex-mix. The amount of solvent to be used is dependent on the size of the analysis cuvette. For instance, use 250µL of solvent when using a 200µL cuvette. The excess solvent allows the sample to be extracted from the tube without disturbing the blanched microspheres in the pellet at the bottom of the tube.
3. Let tubes to stand for at least 15 min to allow the solvent to completely elute the colored dye from all the microspheres. Vortex-mix the tubes again, then centrifuge the tubes for 5 min at 1,500g to form a pellet of the now blanched microspheres and any remaining debris. The supernate solution above the pellet will be drawn from the sample tube with a Pasteur pipette with bulb for dye analysis. This should be done carefully to avoid disturbing the microsphere pellet at the bottom of the tube as any microspheres in the cuvette can affect the absorbance readings.

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4. Transfer the supernate from the sample tube to the appropriate sized microcuvette (100 $\mu$ L to 200 $\mu$ L). If any of the absorbance-peaks in a sample exceed 1.5AU it will be necessary to dilute the sample down into the linear AU region of the spectrophotometer.

Remove any sample with a high AU reading from the cuvette and place it back into it's 'sample' tube. The sample should then be diluted with an amount of solvent that will make the peak reading slightly less than 1.5AU. Mix the diluted sample by repeated filling and emptying the transfer pipette from the sample tube several times. Next, transfer the diluted sample back into the cuvette. It is very important to keep track of which samples have been diluted and the amount of the dilution. This information will be used in the later calculations of blood flow.

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### 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)

100µL to 200µL UV/VIS glass spectrophotometer microcuvette

Ring-Stand with clamps, bars, etc.

200µL Adjustable Pipetter with disposable tips

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

Vacuum Aspirator (with waste collection reservoir)

Pasteur pipettes with bulbs

50mL Conical Polypropylene Screw-top Centrifuge Tubes (FALCON 35-2098 or equiv.)

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 Homogenizer ( Ultrasonicator' ) 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.)

\* Ultrasonic Processor Footswitch (optional, 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](http://www.Sigma-Aldrich.com)). All reagents should be A.C.S. grade or better.

<b>ETOH</b> - Denatured Ethyl Alcohol, 1 liter .....	<b>Sigma-Aldrich</b>	27,074-1
<b>Tween 80</b> , 25mL .....	<b>Sigma-Aldrich</b>	27,436-4
<b>Triton X-100</b> , 1 Liter .....	<b>Sigma-Aldrich</b>	27,074-1
<b>Potassium Hydroxide</b> , pellets, (FW 56.11, 1kg) .....	<b>Sigma-Aldrich</b>	22,147-3
<b>Hydrochloric Acid</b> , 37%, 500mL .....	<b>Sigma-Aldrich</b>	25,814-8
<b>Sodium Azide*</b> , (FW 65.01), 5 gram .....	<b>Sigma-Aldrich</b>	19,993-1
<b>Thimerosal</b> , (FW 404.8), 1 gram .....	<b>Sigma-Aldrich</b>	E3,525-1
(Ethylmercurithiosalicylic Acid, sodium salt)		
<b>Sodium Chloride</b> , (FW 58.44), 100 gram .....	<b>Sigma-Aldrich</b>	22,351-4
<b>DMF**</b> <i>N,N</i> -Dimethylformide, <i>spectrographic grade</i> , 1 liter ...	<b>Sigma-Aldrich</b>	15,481-4
or		
<b>Cellosolve Acetate**</b> 2-Ethoxyethyl Acetate, 1 liter .....	<b>Sigma-Aldrich</b>	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 Thimerosal are used as bacteriostats in solutions that will be stored for some time.

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

### 1.7 Processing Reagents Recipes:

(store all reagents at room temperature):

#### **Blood Hemolysis Reagent:**

Add 1 liter of 10% Triton X-100 solution (see below) to a 2 liter glass beaker. Place the beaker on a magnetic-stirring plate and begin stirring with a magnetic stirring bar. Add 200mL of Ethanol to the solution with stirring. Store the solution in plastic bottles identified as "**Blood Hemolysis Reagent (BHR)**".

#### **Alkaline Digestion Reagent (1M ADR):**

Add 2000mL of distilled water to a 2-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 112.22 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 "**Alkaline Digestion Reagent (ADR)**". **Caution: This is a very caustic solution. Wear Latex gloves and handle with care !**

#### **Acidified Ethanol Reagent:**

Prepare a stock solution of *acidified ethanol* by adding hydrochloric acid (HCl, 37%) to a beaker of ethanol, 0.2% (volume/ volume). For example, add 2mL of HCL to 1000mL of ethanol. Store the solution in a 1 liter plastic bottle identified as "**Acidified Ethanol (AE)**"

#### **Acidified Cellosolve Acetate:**

Prepare a stock solution of *acidified Cellosolve Acetate* by adding 10 $\mu$ L hydrochloric acid (HCl, 37%/10 Normal) to 100mL of Cellosolve Acetate.

#### **10% Triton X-100 Reagent:**

Add 1800mL of distilled water to a 2-liter glass beaker. Place the beaker on a magnetic-stirring hot plate and heat the water to approximately 50° C. Place a magnetic stirring bar into the water and begin stirring at a fairly rapid rate. Add 0.20 gram Sodium Azide to the distilled water. Add 200mL of Triton X-100 to the hot water and stir the viscous Triton X-100 into water 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 "**10% Triton X-100**".

#### **15% Triton X-100 Reagent:**

Add 1700mL of distilled water to a 2-liter glass beaker. Place the beaker on a magnetic-stirring hot plate and heat the water to approximately 50° C. Place a magnetic stirring bar into the water and begin stirring at a fairly rapid rate. Add 0.20 gram Sodium Azide to the distilled water. Add 300mL of Triton X-100 to the hot water and stir the viscous Triton X-100 in water 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 "**15% Triton X-100**".

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### **10% Tween® 80 Solution:**

Add 90mL of distilled water to a 1-liter glass beaker. Place the beaker on a magnetic-stirring hot plate and heat the water to approximately 50° C. Place a magnetic stirring bar into the water and begin stirring at a fairly rapid rate. Add 0.05 gram Thimerosal to the distilled water. Add 10mL of Tween® 80 to the hot water and stir the viscous Tween® 80 in water 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 "**10% Tween® 80**". Note: The shelf life of this reagent is approximately 2 weeks.

### **0.05% Tween® 80/Saline Solution (Microsphere Carrier Solution):**

Add 0.5mL 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.