User's Manual

for

DYE-TRAK & DYE-TRAK VII+

Measurement of Regional Blood Flow

Utilizing Colored Microspheres

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A. INTRODUCTION

ABOUT THIS MANUAL

This guide provides an overview for the use of Dye-Trak and Dye-Trak VII+ microspheres for measurement of regional blood flow in experimental animals. Dye-Trak microspheres offer a non-radioactive alternative to the previously used radio-isotope labeled microspheres. While every investigator will design his or her own experimental protocol, certain steps in the injection, retrieval and quantification of these spheres will be shared by all the Dye-Trak microsphere users. This guide is principally intended for the investigative personnel responsible for setting up and executing the experimental procedures. The scientific basis for use of non-radioactive microspheres has been thoroughly validated in the literature*.

We welcome your suggestions and comments regarding this guide. Please address any questions or comments concerning this manual to us via FAX at (858)272-1451 or e-mail: *triton2@san.rr.com*. This manual may be revised from time to time as necessary.

OVERVIEW

For over forty years tracer microspheres have been used to measure regional blood flow in animal research models. Tracer microspheres are marked or tagged in some manner that makes them uniquely identifiable. By using several different markers, regional blood flow can be determined at several time points during an experiment.

The basic concepts are straight forward: Marked inert microspheres, of uniform size, approximately twice the diameter of a red blood cell, are injected into the central circulation (usually the left atrium) where they are homogeneously mixed in the blood. Blood then flows through the aorta out to all points in the vasculature. The microspheres, because of their size, cannot pass through the smallest capillaries and are trapped in the tissue beds throughout the entire circulation in direct proportion to the volume of blood flowing to each tissue at the time of the central microsphere injection. This is the basis of the microsphere technique and is true for all the various microsphere marking methods.

The number of microspheres injected, while seemingly a large number, actually block only a very small proportion of the total cross-sectional area of the capillaries and thus has no discernable effect on muscle or organ function as has been shown in the literature *. The number of microspheres found in any tissue bed at the end of an experiment will be directly proportional to the volume of blood flowing to the tissue at the time of the central microsphere injection. Due to the mixing in the left atrium/left ventricle there is some latency and therefore several cardiac cycles must pass before all the microspheres from an injection will pass out into the aorta. The importance of this fact will be covered in the discussion on the measurement of volume blood flow.

***Kowallik, P., R. Schulz, B. D. Guth, A. Schade, W. Paffhausen, R. Gross and G. Heusch. Measurement of regional myocardial blood flow with multiple colored microspheres. Circulation 83:974-982, 1991.**

B. REGIONAL BLOOD FLOW MEASUREMENT

RECENT BACKGROUND- RADIOACTIVE MICROSPHERES

Throughout the 1970's and 1980's there were large numbers of regional blood flow studies done using microspheres marked with one of several radioactive isotopes. There were a number of troubling environmental health and safety concerns associated with the use of radioactive isotopes. There were however several thousand papers and abstracts published on a very wide range of applications in many different animal models using radioactive microspheres. New England Nuclear and 3M were the principle suppliers of radioactive microspheres. Before they stopped supplying radioactive microspheres, 3M published a very comprehensive bibliography of the radioactive microsphere literature in 1987, categorized by organ and species (contact Triton Technology for copies of the 3M bibliography).

There were a number of good and bad features or the use of radioactive isotopes. A good feature of radioactive microspheres was the fact that the radio activity could be counted without recovering the microspheres from the tissue samples. The tissue samples could simply be placed in a test tube and run through an automated gamma counter, as the radioactivity could be 'counted' directly from the intact samples. This feature made the processing of the tissue and blood samples very simple. There were issues of radioactive spill-over between the various isotopes, but this was easily handled with matrix inversion compensation using the *a priori* knowledge of each isotope's radiation spectrum.

The biggest problem with the radioactive microspheres was the environmental health and safety issues. Because of these issues there were special licensing and record-keeping requirements associated with the use of radioactive microspheres. Radioactive microspheres had to be kept in lead shielding and kept away from direct human contact. Users had to wear film badges to allow periodic checking for radioactive exposure.

Radioactive animal carcass and sample disposal issues were also major problems. In order for the radioactivity to decay to an acceptable level, carcasses had to be held for long periods of time before they could be disposed of by normal means. While working with mice, rats, rabbits and other small animals, this was not a big problem. It was a big problem when working with larger animals such as dogs or pigs. The carcasses had to be stored in freezers for many months prior to disposal. At many institutions this resulted in hallways being lined with top-load freezers full of carcasses awaiting disposal. The animal waste products also had to be considered to be potentially radioactive and it was necessary to handle and store waste as if it were radioactive.

Radioactive isotopes continuously lose activity with time as a result of radioactive decay. Because of this, radioactive microspheres have a limited shelf life. If not used in a timely manner, radioactive microspheres will become useless after a relatively short period of time due to radioactive decay. Likewise, once the radioactive microspheres have been injected into an animal they continue to loose radioactivity with time, thus limiting the length of chronic animal studies.

The radioactive microspheres are dense and thus may not behave like red blood cells in the circulation. Some authors feel that this density difference causes non-physiologic changes to blood flow distribution measurements.

NON-RADIOACTIVE MICROSPHERES

All of the negative features of radioactive microspheres lead to the development of non-radioactive tracer methods for the measurement of regional blood flow. Markers other than radioactive marking required the recovery of the microspheres from the tissue for counting. Microsphere recovery is accomplished by 'digesting' the tissues in potassium hydroxide (KOH) and recovering the microspheres by filtration or centrifugation. A number of tracer marking techniques have been tried in order develop non-radioactive microspheres for regional blood flow measurements. The principle approach has been to use colored dyes or fluorescent dyes to mark the microspheres. One of the early attempts to develop non-radioactive microspheres involved color dyeing and subsequent visual counting of the microspheres recovered from the tissue samples.

Due to the large number of microspheres in a sample actually only a small aliquot of the sample was counted, either visually under a microscope or in some video-automated way. The counts from the small aliquot were then mathematically extrapolated to come up with a value for the entire sample. This was a very labor intensive technique with questionable accuracy and it never found much application.

Another approach was a semi-automated counting method using specially adapted fluorescence flow cytometers to count the various marked microspheres in a sample.

The flow cytometer technique and other specialized microsphere types have been used for a 'mail-in' tissue processing services, where experienced technicians processed the user-supplied tissue and blood samples for a fee. These 'mail-in' type services have never become very popular among users for several reasons:

1) The time delay in getting tissue and blood sample results returned makes it impossible to use the information from one experiment to modify the next experiment.

2) There is a loss of control of the material. Samples can be lost either in transit or at the processing lab. Likewise the potential for samples being mishandled or mixed is always a concern.

3) Contract processing is expensive when doing a large number of samples and there are additional costs for computing volume blood flow from the tissue samples.

The dye-elution technique with tissue processing and microsphere recovery is not difficult to master. Quick access to measurement results makes it possible to modify ongoing experimental protocols as new experimental results become available.

DYE ELUTION TECHNIQUE

By far the most successful technique for non-radioactive microspheres is to dye polystyrene latex microspheres with either absorbent or fluorescent dyes. As with all of the microsphere types, the microspheres are injected into the central circulation in order to achieve a homogeneous distribution of the microspheres in the circulating blood. At the conclusion of the experiment, the animal is sacrificed and tissue samples from the organs, muscles or other tissues of interest are harvested and weighed while fresh. It is important to weigh and record the fresh tissue samples as this 'wet' weight will be used later to determine volume blood flow per gram of tissue.

The harvested tissue samples are then digested in a strong base solution (KOH) and the microspheres recovered by either filtration or centrifugation. The dye is extracted from the recovered microspheres for each tissue sample using a known volume of a specific solvent. For the absorbance-dyed microspheres, the absorbance spectrum of the solvent solution for each tissue or blood sample is then analyzed on a spectrophotometer in order to determine the quantity of dye of each color in each tissue sample. In a similar manner, the extracted dye solution from fluorescent microspheres is analyzed on a fluorescence spectrophotometer ('fluorimeter'). The quantity of dye of each color in a tissue sample is a measure of the number of microspheres of each color in the sample and thus the portion of the total cardiac output going to this tissue sample at the time that color of microsphere was injected..

It is important to note that with the dye elution technique, the samples need not be analyzed immediately. This is a major advantage over the radioactive microspheres which must be analyzed soon because of the continuing decay of the radioactive isotopes in the microspheres with time. By contrast, the dyed microsphere tissue and blood samples can be stored indefinitely (preferably in capped tubes in a refrigerator). Further, the recovered microsphere samples or even the extracted dye samples can also be stored indefinitely. Extracted dye samples can be kept archived under refrigeration for long periods of time should it be necessary to re-analyze a sample to verify data. Recovered microsphere samples or extracted dye samples should not be exposed to intense light and the extracted dye samples should be stored in capped sealed tubes to prevent evaporation from increasing the concentration of the dyes in the samples.

DYE-TRAK DEVELOMPENT

The dye elution method is the basis for a number of patented microsphere products including Triton's Dye-Trak, Dye-Trak VII+ and Dye-Trak 'F' fluorescent microspheres and also the Molecular Probes 'FluoSpheres' fluorescent microspheres. The dye elution approach was pioneered and patented by Triton Technology in the early 1990's working with researchers at the University of Essen in Germany and also with Bayer Pharmaceutical Company in Germany. Triton Technology was issued several US and European patents for the dye-elution microsphere technique. Triton Technology markets both absorbance-dyed and fluorescence-dyed microspheres of our own manufacture.

In addition, Triton Technology licenses its patents to Molecular Probes (Invitrogen) for them to sell their own line of fluorescent microspheres based on their own proprietary dyes. (Molecular Probes *FluoSpheres* are available from Triton Technology at discounted prices.) The Triton Technology Dye-Trak 'F' fluorescent microspheres meet or exceed the performance of the Molecular Probes FluoSpheres for about half the price of the Molecular Probes microspheres.

DYE-TRAK MICROSPHERE CHARACTERISTICS

All of the Triton Technology microspheres (Dye-Trak , Dye-Trak VII+ and Dye-Trak 'F' fluorescent microspheres) are made with polystyrene latex, cross-linked with 2% DVB **(**divinylbenzene). These microspheres are 15µm in diameter and are accurately sized with a standard deviation of 0.1µm. The density of the Dye-Trak microsphere is approximately 1.09, thus the Dye-Trak microspheres will slowly settle in water or normal saline. Thus the Dye-Trak microspheres have settling rates similar to red blood cells and are thought to behave similarly to red cells in the circulation.

The Dye-Trak microspheres are very stable in an aqueous environment and are not affected by pH. The microspheres can remain *in vivo* indefinitely with no degradation. Properly stored, the Dye-Trak microspheres have a very long shelf life. We recommend keeping the microspheres in a cool, dark place. A refrigerator at 4°C is an ideal storage place. We do not recommend freezing the microspheres, as there is always the possibility the microspheres will fracture and split apart.

The various color dyes are very tightly held within the body structure of the microspheres and the dye is only released when the microspheres are swollen by exposure to any one of several of organic solvents. In addition, the microspheres should not be exposed to hot ETOH, as this too will tend to release some of the dye from the microspheres.

The *original* Dye-Trak absorbance-dyed microsphere family is dyed with one of five colors: White, Yellow, Eosin, Violet and Blue.

The second generation Dye-Trak VII+ family of colors is available in the following colors: Lemon, Yellow, Orange, Tangerine, Persimmon, Berry and Blue. This family has more colors with stronger absorbance peaks (higher AU per microsphere) than the original Dye-Trak colors and sharper peaks with less spill-over between colors.

Similar to the radioactive microspheres, the absorbance microspheres exhibit *spill-over* between the adjacent colors. Spill-over means that the absorbance spectrum of an individual color also contributes some absorbance at the absorbance peak of one or more adjacent colors. Similar to the radioactive microspheres, the spill-over can be mathematically corrected using the *a priori* knowledge of the spill-over characteristics of each color. A mathematical process known as 'matrix inversion' can be used to remove the spill-over component from the individual color peak absorbance readings. Using knowledge of the spectrum of each of the individual colors, the matrix inversion process determines what the peak absorbance would have been without spill-over for each of the colors used in an experiment. Triton Technology supplies free Microsoft Excel macros for volume blood flow computations that incorporate the matrix inversion function.

Measurement Wavelength	Lemon	Yellow	Orange	Tangerine	Persimmon	Berry	Blue
390	**100**	27	1	3	2	9	2
440	1	**100**	8	0	0	14	1
495	2	1	**100**	34	17	10	3
525	1	1	0	**100**	45	26	7
545	1	1	1	32	**100**	50	13
590	1	1	1	0	0	**100**	40
670	0	0	0	0	0	0	**100**

TYPICAL DYE-TRAK VII+ SPILLOVER MATRIX

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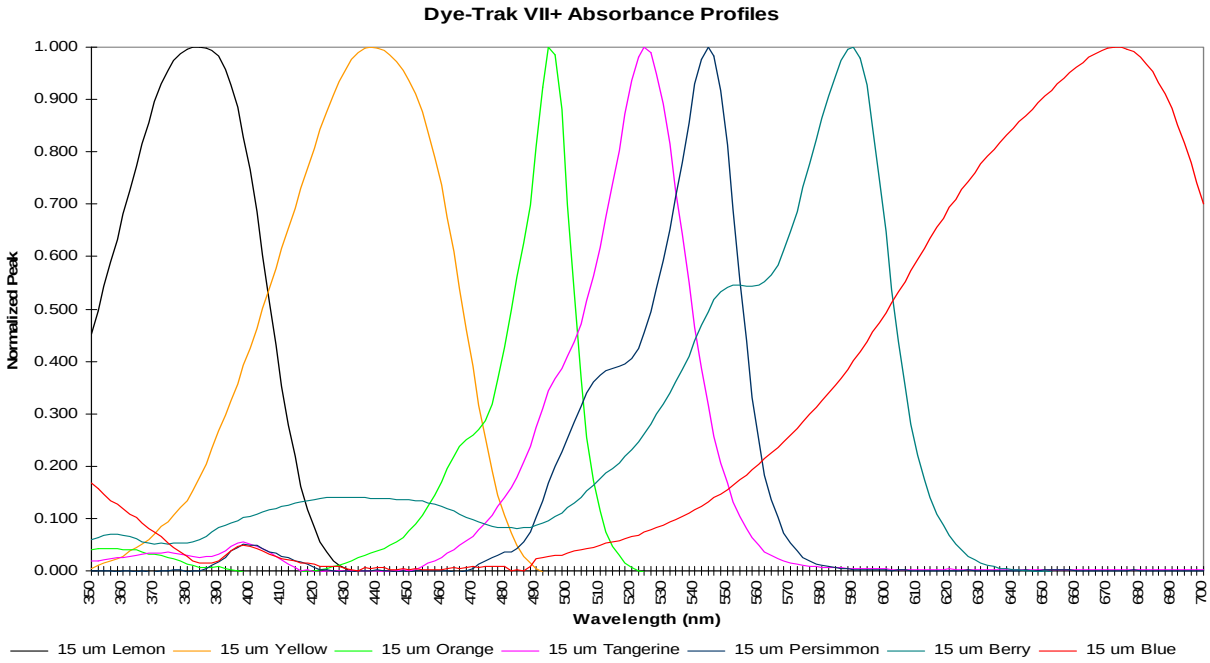


TYPICAL DYE-TRAK VII+ ABSORBANCE CHART

BIBLIOGRAPHY

Since the early 1990's many hundreds, if not thousands of papers have been published using the dye elution method of regional blood flow measurement using dyed microspheres for absorbance or fluorescence quantification. An excellent on-line bibliography of papers utilizing non-radioactive microspheres to quantify regional blood flow is maintained by the University of Washington 'Fluorescent Microsphere Resource Center' or FMRC.

Even though the main thrust of the FMRC web site is theory and use of fluorescent microspheres, the bibliography covers publications for both absorbance-dyed and fluorescence-dyed microspheres

http://fmrc.pulmcc.washington.edu/WebRefs.shtml

C. THEORY OF VOLUME BLOOD FLOW MEASUREMENT

Recovering the microspheres from various organs and tissues allows the user to determine relative blood flow in the various tissue samples by comparing the number of microspheres in each tissue sample to all the other tissue samples taken from the animal on a 'per weight' basis. Volume blood flow can be determined by adding an additional 'reference withdrawal' step during the injection of each microsphere marker. This reference withdrawal step for volume blood flow measurement is the same for all microsphere types (radioactive, fluorescent, absorbent, etc)

REFERENCE BLOOD SAMPLE

As each experimental microsphere 'color' is injected into the left atrium (or left ventricle in small animals), a sample of blood is withdrawn from the descending aorta or another artery downstream of the aortic valve. This reference withdrawal blood sample is taken at a known rate using a previously calibrated motorized syringe pump. The withdrawal begins before the injection and continues for a while (perhaps a minute) after the injection to insure that all the microspheres in the aortic blood are represented in the blood sample, including those that remain in the atrium and ventricle for several cardiac cycles. The amount of blood withdrawn does not affect the volume blood flow calculation, as it is the rate of withdrawal that is the value that is used in the calculation of volume blood flow.

As with the tissue samples, each blood sample is digested in potassium hydroxide (KOH) to recover these reference microspheres. The reference withdrawal syringe rate must be accurately calibrated and the rate value recorded, as the withdrawal rate becomes part of the volume blood flow calculation. The withdrawal syringe rate should be calibrated by 'beaker and stop watch' method prior to use in the experiments. Glass rather than plastic syringes are recommended for use with the withdrawal syringe pump to insure repeatability.

Rather than 'count' the microspheres in each tissue or blood sample, the dye trapped in the microspheres is recovered and analyzed spectrographically. The amount of dye of each experimental color in each sample is directly proportional to the number of microspheres in the sample (tissue or blood). Each tissue sample should be weighed while fresh and still wet, before it is digested. The amount of dye of the color injected in each blood reference withdrawal sample is used as the denominator of the volume blood flow calculation, while the amount of dye of that color in each tissue sample is used in the numerator of the blood flow calculation. Using the wet weight of each sample, the volume blood flow in ml/minute/gram can be calculated.

REGIONAL BLOOD FLOW CALCULATION

Volume blood flow using absorbance-dyed microspheres is computed by a simple ratio calculation:

$$\frac{AU(blood)}{RATE(blood)} = \frac{AU(sample)}{RATE(sample) \times WEIGHT(sample)}$$

$$RATE(sample) = \frac{[AU(sample)] \times [RATE(blood)]}{[AU(blood)] \times [WEIGHT(sample)]} \quad (\text{ml/min./gram})$$

Where:
AU(blood) = Absorbance Units for **Reference Withdrawal** blood sample
AU(sample) = Absorbance Units in a given tissue sample
RATE(blood) = Withdrawal rate for the **Reference Withdrawal** blood sample
RATE(sample) = Volume of blood flow for the sample (ml/min/gram).
WEIGHT(sample) = Wet-weight of freshly harvested tissue sample (grams)

Here it is assumed that the volume of solvent used is the same for both the tissue and blood samples. If this is not the case, then the different volumes of solvent used must be accounted for in the volume blood flow calculation.

By using the weight of the freshly harvested tissue samples, i.e.'wet weight', the resultant volume blood flow for each sample can be expressed in units of ml/minute/gram

END ORGAN BLOOD FLOW MEASUREMENT

It is also possible to measure relative flow distribution within an end-organ, such as the kidney or heart, by injecting microspheres into the blood vessel feeding the end organ. The microspheres should be injected as far upstream as possible from the end organ to insure homogeneous mixing of the microspheres within the circulating blood. The microsphere injection should always be immediately followed with a saline flush of sufficient volume to clear all the microspheres from the injection catheter. Microspheres should appear in all locations of the end organ in direct proportion to the blood flow at the moment of injection. Sampling various portions of the end organ will provide relative blood flow comparisons between various areas within the end organ. All tissue samples should be weighed while still fresh (wet weight) and that information recorded so that the relative blood flow measurements for different locations in the organ can be normalized for tissue weight.

The relative blood flow information can be converted into volume blood flow per gram of tissue by the addition of a volume blood flow meter on the perfusing vessel in order to measure the total flow perfusing the end organ.

$$\frac{\text{Total AU (injected)}}{\text{Measured Flow}} = \frac{\text{AU(sample)}}{\text{RATE(sample)} \times \text{WEIGHT(sample)}}$$

$$\text{RATE(sample)} = \frac{[\text{AU(sample)}] \times [\text{Measured Flow}]}{[\text{Total AU (injected)}] \times [\text{WEIGHT(sample)}]} \quad \text{(ml/min/gram)}$$

Where:

Total AU (injected) = Total AU injected *
AU(sample) = Absorbance Units in a given tissue sample per solvent volume
Measured Flow = Volume blood flow measured by flow meter on perfusing vessel (ml/min)
RATE(sample) = Volume of blood flow for the sample (ml/min/gram)
WEIGHT(sample) = Wet weight of freshly harvested tissue sample (grams)

** The total AU injected should be determined by the user on his/her own spectrophotometer by first measuring a large number of microspheres (i.e. 5000 per 100µl of solvent) in order to determine the number of AU per ml for each color to be injected in the end-organ experiment.*

By using the weight of the fresh tissue sample, (wet weight), the resultant volume blood flow can be expressed in units of ml/minute/gram.

PROCESS CONTROL

Process Control is an inexpensive method of detecting the loss of microspheres during the tissue digestion and microsphere recovery from the digested tissue and blood samples. Microspheres can be lost by accidental spills, improper handling, poor technique, etc during the various processing steps in the tissue digestion/microsphere recovery.

Process Control involves placing a known number of Blue microspheres into each tissue or blood sample tube prior to the digestion and microsphere recovery steps. The Blue microsphere colors are used for Process Control because none of the other colors has spill-over into the Blue spectral peak, thus the 670nm reading is only due to the presence of the Blue microspheres. A repeating pipette is used to draw the Process Control suspension from a constantly stirred 'stock solution' of Blue microspheres. Since all the tissue and blood sample tubes have the same number of Blue microspheres in them, there should be the same amount of Blue dye in each sample's recovered microspheres. Thus the measured results for each individual tissue or blood sample will indicate if any microspheres were lost during processing. Triton Technology supplied software will then calculate a 'percent recovery' value for each sample as an indicator of good lab technique.

The Triton software then calculates the 'correct' AU values for each of the experimental color peaks in the sample. A reduced amount of Blue dye in a sample will indicate that microspheres were lost from that sample during processing. The *loss-corrected* absorbance values for each sample are then processed by matrix inversion and then used to calculate volume blood flow for each sample.

Typically 10,000 Blue microspheres will be placed into every tissue sample or blood tube with a repeating pipette prior to beginning the digestion process. A stock solution of Blue microspheres can be prepared for this purpose by diluting Blue spheres in a saline solution containing 0.05% Tween 80. It is necessary to have at least 0.01% Tween 80 in the dilution solution in order to prevent the microspheres from sticking together and clumping. The recipe for the 0.05% Tween 80 solution can be found in both the FILTRATION or SEDIMENTATION tissue processing procedures sections of this manual. To prevent the microspheres from settling, the Process Control solution should be constantly stirred with magnetic stir bar while it is being used.

When you do the spectral analysis on the dye extracted from the recovered microspheres in a sample, you should find an amount of Blue color that is the equivalent of 10,000 Blue spheres in each sample. If there is less Blue dye in a sample, then microspheres were lost in processing. Process Control will also detect if a sample was diluted with more solvent than intended, as this will proportionately decrease the measured Absorbance units (AU) for each and every color in the sample.

The Process Control version of our free analysis software will automatically back-calculate the correct value of all the colors in a sample, as if no spheres were lost. Our tests have shown that even with large losses of microspheres, you will arrive at the correct values for each sample. The cost for Process Control is only a few cents per sample. For instance, one 2ml bottle of Blue (containing six million spheres) will make up enough Process Control solution to do many tissue samples.

<u>We strongly recommend using Process Control.</u>

VOLUME BLOOD FLOW CALCULATION SOFTWARE

For users of the Dye-Trak and Dye-Trak VII+ absorbance-dyed microspheres, Triton Technology supplies free Microsoft Excel spreadsheet macros which utilize the user-supplied data to calculate volume blood flow for each tissue sample. The user must supply the following data:

- Absorbance values for each sample of tissue or blood
- The tissue wet-weight for each tissue sample.
- Single color standards for each color used in the experiment.

The software macros are designed to automatically transfer the absorbance data files from most major brands of spectrophotometer directly into the spreadsheet, thereby decreasing the chance of user error entering the data. Triton will modify the macros for automatic data transfer for new file structures as necessary to support newer spectrophotometers. Please contact us if the macros will not read the ASCII data files from your spectrophotometer.

There are several versions of the software designed for both the *original* 5-color Dye-Trak and also the newer 7-color Dye-Trak VII+ family. Further, there are versions of the software for use with and without a Blue Process Control. Triton strongly recommends the use of Blue Process Control microspheres. For a cost of a few cents per sample, the user will be aware of the recovery efficiency for each processed sample or the loss of microspheres in processing or even an error in solvent volume used. The macros then use the recovery factor to correct the measured AU values for all the experimental colors to what they would have been with 100% recovery. The software corrects for all amounts of microsphere loss, small or large.

Instructions and notes on the use of the Triton Technology free Microsoft Excel spreadsheet macro can be found in **Appendix B** of this manual. **Appendix B** can best be viewed in color, or printed on a color printer, sothe colored cells of the spreadsheet can be seen as they appear on the computer.

D. NUMBER OF MICROSPHERES TO INJECT

OVERVIEW

The quick answer to how many microspheres to inject can be found by researching the literature in order to determine the number of microspheres other investigators have injected in similar animal models. Some of the Dye-Trak microsphere colors have a stronger 'signal' than others and this should be taken into consideration when determining the number of microspheres of each color to inject. Also when doing low-flow or ischemic blood flow studies, more microspheres will need to be injected in order to get useful AU readings.

Buckberg, et al*, working with radioactive microspheres, looked at the statistical requirements for accurately measuring blood flow using microspheres. This work is also directly applicable to all microsphere types including dyed microspheres of either the fluorescent-dyed or absorbance-dyed types. Buckberg concluded that it is necessary to have at least 400 microspheres in each sample in order to have a statistically significant result. 400 microspheres should be considered as an absolute minimum number to be found in a sample.

It is important to note that this 400 microsphere minimum number is independent of the sample size. In other words, if you are working with 2 gram samples, you want at least 400 microspheres in the sample. If you are working with 1gram samples, you still want to have at least 400 microspheres in the sample, etc. While 400 microspheres per sample is the minimum, as a safety factor, users should design experimental protocols so that tissue samples with the lowest flow, have at least 800 microspheres per sample.

As an example, for an end-organ study of the kidney, microspheres will be injected into the renal artery. The microspheres should be injected in a number sufficient that any and all the tissue samples from the end organ will meet the 400 microsphere per sample minimum for statistical significance. Given that the kidney to be studied weighs approximately 150 grams and you plan to measure 1 gram tissue samples, then you should inject at least 150 x 400 spheres into the renal artery. That way if there is equal perfusion to all parts of the kidney, you could take any one gram sample and assume you would find at least 400 microspheres. To be on the safe side you should inject even more microspheres; the goal being not only to exceed the 400 microspheres per sample, but also insure that the AU readings for the samples with the lowest blood flow will be not only above the statistical threshold, but also well above the spectrophotometer's baseband noise and detection threshold.

The 400 microsphere minimum for statistical sampling criterion is only part of the story. Spectrophotometer baseline noise, minimum detectable signal threshold, and absorbance linearity are the other important issues to be considered. The minimum detectable signal on a spectrophotometer is around 0.05AU. This is assuming that the spectrophotometer has been baseline corrected across the entire spectrum with a cuvette containing only solvent (DMF or *acidified* Cellosolve Acetate). Signals below 0.05AU are not considered reliable indicators of absorbance for blood flow applications. Because the spectrophotometer readings become progressively more non-linear above about 1.3AU, it may become necessary to further dilute some samples if the absorbance values for any of the colors exceeds 1.3AU. When this happens, the sample will need to be diluted with an additional known amount of solvent in order to bring the highest peak AU reading into the linear region. The information on any subsequent dilutions to any sample should be recorded along with the sample number for use in making the final blood flow calculation.

EMPIRICAL APPROACH

Another way to determine the number of microspheres to use in your animal preparation is to do a test animal, and process the samples from tissues of interest to determine if sufficient number of microspheres were injected to give useable absorbance data for each color in a normal flow situation.

***Buckberg, Gerald, et al, "Some sources of error in measuring regional blood flow with radioactive microspheres", J. of Applied Physiology, Vol. 31, No. 4, October 1971** (contact a Triton Technology for reprint**)**

MICROSPHERE MEASUREMENT OF REGIONAL BLOOD FLOW

If you plan to do low flow or reduced blood flow studies, then it will be necessary to increase your final microsphere injection numbers in order to account for the lower flow in some samples. When doing test animals, it is important to take the samples from the organs of interest in the same size that will be used in the actual experiments or if you take different size test samples, correct for this fact when you determine your final injection numbers.

When preparing to do a regional blood flow study, a user should review the published literature for the animal model of interest to determine what percentage of the cardiac output goes to the organs of interest. Those percentages of the cardiac output (and thus the microspheres injected into the left atrium) will be going to those organs. Next, determine the size of the proposed tissue samples, expressed in terms of percentage of the organ weight. For instance, you intend to take one gram samples of a 200 gram heart which receives 5% (or 1/20) of the cardiac output. You will need to have at least 400 microspheres in any one gram of tissue taken from this heart for statistical reasons.

Based on these assumptions, you would need to inject at least 1.6 million microspheres of each experimental color. This might be your first "best guess" number to inject.

$$\frac{200\ \text{gm}}{1\ \text{gm}} \times 400\ \text{microspheres} \times 20 = 1{,}600{,}000\ \text{microspheres}$$

This only addresses the statistical question, but not the variation in signal strength between colors. Just as with radioactive microspheres, each of the colors has a different 'signal strength' and this should be considered when determining the number of microspheres to inject.

One approach for doing the first test animal is to make up a test injection 'cocktail' with a mix of all the colors you intend to use and make just one injection. By doing one injection, you can assume that the blood flow to each tissue sample will be the same for each color injected. After the injection, the animal can be sacrificed and the representative tissue samples harvested, the tissues samples processed, the microspheres recovered and the extracted dye analyzed on the spectrophotometer. This initial cocktail mixture could be made up with the ratios of the various colors in the approximate ratios listed below for the first test injection: These ratios correspond to approximately equal numbers of absorbance units (AU) for each color within the two Dye-Trak families.

DYE-TRAK	
Eosin	1.0
Yellow	1.1
White	2.0
Blue	2.6
Violet	3.7

DYE-TRAK VII+	
Orange	1.0
Persimmon	1.1
Yellow	1.2
Tangerine	1.2
Lemon	2.2
Berry	2.7
Blue	2.7

The minimum useable absorbance signal on the spectrophotometer is approximately 0.05AU. The absorbance readings on the spectrophotometer become non-linear for readings above about 1.3 AU. The goal on the test animal(s) is to achieve AU reading in the linear region of the spectrophotometer for all tissue and blood samples. After doing one or more test animals the ratio of the colors to inject and the absolute number of each color to inject can be determined.

ADDITIONAL CONSIDERATIONS:

The useful range of a UV-VIS spectrophotometer could be considered to be between 0.2AU to 1.3AU. Thus a sufficient number of microspheres of each color must be injected to have all normal flow readings fall in the 0.2 to 1.3 AU region. In addition, if low flow studies are going to be conducted, then the numbers of microspheres to be injected must be increased in order to insure that absorbance readings even for low flow samples will still be above 0.2 AU.

Because of the potential for instrument errors, a sufficient number of microspheres should be injected so that each of the tissue samples will have AU readings that fall in the desirable 0.25AU to 1.25 AU range. If any of the colors in a tissue or blood sample shows an absorbance above 1.3 AU, the entire sample will need to be further diluted with a known amount of solvent and the absorbance re-measured. The dilution factor of any samples that are further diluted will need to be recorded along with the sample number for later use when doing the blood flow analysis on that particular sample.

There is a short internet article discussing the potential errors in UV-Visible spectrophotometry titled **Performance of UV-Vis Spectrophotometers** by Dr. Herman Lam which summarizes areas of potential error using UV-Visible spectrophotometers. Users who are new to the use of UV-Vis spectrophotometry might consider downloading and reading this short paper:

(www.cvg.ca/images/performance_UV_VIS.pdf)

When fabricating the Dye-Trak and Dye-Trak VII+ microspheres, Triton Technology strives to put as much dye as possible into each microsphere. Because of the inherent characteristics of each of the dyes, the different colored microspheres have different amounts of absorbance per microsphere. This factor should be taken into consideration and compensated for by injecting more microspheres of the colors with low AU reading and fewer microspheres of the colors that have high AU readings.

The investigator can use this variation in absorbance verses color when selecting which color to inject at different time points in an experiment. This is particularly true in experiments that include normal flow and low flow tissues. The high absorbance colors can be used for low-flows and the lower absorbance colors used for normal flow tissue.

The absorbance for each color is usually expressed as 'AU per 5000 microspheres per 100µl of solvent' or AU/5000/100µl. The absorbance values for the various colors range from about 0.45AU to 1.8AU per 5000 microspheres per 100µl of solvent. Listed below are some typical absorbance values for the Dye-Trak and Dye-Trak VII+ families The variability of absorbance between the different colors can be seen:

TYPICAL DYE-TRAK

COLOR	nm	AU/ 5000/100µl	Microspheres/ AU/100µl
White	370	0.869	5754
Yellow	445	1.530	3268
Eosin	530	1.700	2941
Violet	595	0.461	10846
Blue	670	0.675	7407

TYPICAL DYE-TRAK VII+

COLOR	nm	AU/ 5000/100µl	Microspheres/ AU/100µl
Lemon	390	0.851	5875
Yellow	445	1.530	3268
Orange	495	1.830	2732
Tangerine	525	1.550	3226
Persimmon	545	1.660	3012
Berry	590	0.683	7321
Blue	670	0.675	7407

These are example numbers and are shown here only to indicate the relative absorbance of the various colors. All spectrophotometers vary somewhat in their measurement output for a given sample. Users should validate each new batch of microspheres on their own spectrophotometer prior to using the microspheres in an experiment. This would normally be done while doing 'single color' standards for use with the free Triton Technology supplied Microsoft Excel spreadsheet macros. This is the data that is used for spill-over correction.

Typically 100µl of solvent is used when analyzing Dye-Trak and Dye-Trak VII+ microspheres from experimental samples. If you plan to use a larger volume of solvent for the sample dye analysis, this should also be factored in when determining the number of microspheres to inject.

It is not a simple matter of injecting X number of microspheres into an animal that weighs Y kilograms. Prior to starting to do experiment animals, it is critically important to determine how many microsphere of each experimental color should be injected. This should be done so that no experimental animals are needlessly wasted. It is important to analyze the samples from the initial experimental animals before doing too many animals in order to ensure good results and allow the user to adjust the number of microspheres of the various colors to inject in the later experimental animals. The user should always be prepared to change the number of microspheres injected based on the results of prior experiments.

E. ANALYSIS SOFTWARE

Triton Technology supplies free Excel software macros for the blood flow analysis that sequentially open and import data from archived ASCII data files. These macros will import the data files from most brands of spectrophotometer. After the user supplies single-color spectra for all the experimental colors used, the macro performs a matrix inversion calculation which removes all the spill-over between the various Dye-Trak colors. The result of this computation is that each color peak will now be corrected from any spill-over absorbance from adjacent colors. Next, working with the user-supplied tissue weights and Reference Blood information, the Excel macros will calculate blood flow for each tissue sample in ml/minute/gram of tissue.

There are specific macros for each family of Dye-Trak microspheres: *original* 5-color Dye-Trak and the 7-color Dye-Trak VII+

In addition, there are versions for each number of colors used in an experiment. This is because using software for the maximum number of colors in a family and forcing in zero AU reading for unused colors will result in a 'noisier' calculation than if the correct software for the specific number of colors is used.

There are also versions for the free Excel software for use with or without a Process Control color. Using Blue microspheres as a Process Control standard will allow the Excel software to detect the loss of any microspheres during tissue digestion and microsphere recovery. Further, the software can correct the blood flow results for any lost spheres. In this manner, the results will be the same as if none of the microspheres was lost!

Process Control is accomplished by using an accurate repeating pipette to place a known number of Blue microspheres into each tissue and reference blood tube prior to digestion and microsphere recovery. Blue is used because it has the longest wavelength peak of all the colors and none of the other colors spill any absorbance onto the Blue peak. Typically 10,000 Blue microspheres are placed into each sample tube, taken from a stock solution that is constantly stirred with a magnetic stir bar while process control microspheres are being withdrawn into the repeating pipette. The stock solution should be made up using the 0.05% Tween 80 solution. The Tween solution is used to prevent the Blue microspheres from clumping or sticking together.

Contact Triton Technology for information on the free software Excel compatible macros (triton2@san.rr.com).

F. TISSUE PROCESSING - OVERVIEW

At the completion of the experimental portion of the animal study, the animals are terminated and the tissue samples harvested. The fresh samples should be weighed and placed in code-identified screw-top test tubes. The sample tubes should be marked with an identification code and this data should be recorded along with the wet weight, sample location and any important information related to the sample. The tissue and blood samples can be stored in screw-top test tubes at 4°C for long periods of time. Samples can even be kept at room temperature, but these samples will need to be handled under a fume hood when the caps are removed due to the strong odor. The reference withdrawal blood samples must be anti-coagulated as they are drawn with the withdrawal syringe pump, as clotted blood samples can be more difficult to digest. The samples should always be kept in screw-top tubes to prevent them from drying out, as this makes tissue digestion more difficult. The tissue and blood processing and microsphere recovery steps can be suspended at any time during the process and the samples stored in screw-top test tubes under refrigeration for later processing.

The processing can be paused at any point in the tissue processing and microsphere recovery. The recovered microspheres can be stored in screw-top tubes. The individual eluted dye samples can also be stored in screw-top test tubes for later re-analysis. It is important that the eluted dye samples be kept in closed tubes to prevent evaporation.

There are two principle ways in which the tissue and blood samples can be processed and the microspheres recovered: Filtration or Sedimentation.

With the Filtration procedure, the tissue samples are digested in 4M KOH. The microspheres have a density of approximately 1.09 and they will not settle in the dense 4M KOH solution that is utilized in the filtration procedure, nor can they be centrifuged to the bottom of the test tube in 4M KOH. The only way to recover the microspheres in this dense solution is by filtration.

The microspheres can be recovered by sedimentation from a less concentrated KOH digestion solution. The sedimentation process uses 1M KOH digestion solution. Because of the lower density of the 1M KOH the microspheres can be centrifuged to the bottom of the centrifuge tubes.

Filtration and **Sedimentatio**n tissue processing procedures each have advantage and disadvantages. We recommend that new users read through both procedures and select the method that best suits their needs.

G. TISSUE PROCESSING - FILTRATION

This procedure is an updated 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. The instructions for preparing all the required reagent solutions can be found in the **Reagents Recipes** section at the end of this procedure.

This **Filtration** procedure can be used for both the *original **5-color*** **Dye-Trak** microspheres or ***7-color*** **Dye-Trak VII+** microspheres. The significant difference for tissue processing purposes 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.

The *acidified* Cellosolve Acetate must be used for Dye-Trak VII+ as some of the dyes are critically pH sensitive and are affected by any carry over of KOH from the tissue digestion steps. The *acidified* Cellosolve Acetate should be made using the recipe in the Reagent Section of this procedure.

Original Dye-Trak Colors:

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

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

Color Selection Sequence Table for the *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**

*Note: *We recommend that White be read at 390nm, rather than its peak absorbance (370nm) to minimize biologic UV baseline background noise. If your tissue samples do not contain high amounts of UV background absorbance, you might want to read White at the peak absorbance wavelength of 370nm. The Triton Technology data processing Excel macros will need to be modified to read White at 370nm.*

.Dye-Trak VII+ Colors:

Lemon (390nm), **Yellow** (440nm), **Orange** (495nm), **Tangerine** (525nm), **Persimmon** (545nm), **Berry** (590 nm), **Blue** (672nm)

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

Color Selection Sequence 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 its peak absorbance of 370nm in order to minimize biologic UV background noise.

TISSUE PROCESSING – FILTRATION

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 by the Triton supplied Excel blood flow analysis software to correct the measured results for each tissue or blood sample.

Process Control *microspheres cost a few cents per tissue sample!*

If the amount of Blue 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 to the value that would have been measured had there been no loss. The free Triton Technology Excel volume blood flow calculation macros will detect and correct for any of microspheres for all the readings in an given sample.

Process Control is accomplished by using a repeating pipette to add a fixed number of Blue microspheres (typically 10,000), to each sample tube prior to the tissue digestion steps for each of the tissue and blood sample tubes. This can be done by adding 100µl of the Blue Process Control microspheres solution (containing 100,000 Blue microspheres per ml) into each tissue or blood sample tube. The Process Control solution should be constantly stirred with a magnetic stir bar while this is being done.

Blue microspheres are used for this purpose because the Blue color is at the long-wavelength end of the spectrum and none of the other colors spill over into the Blue absorbance wavelength peak. The instructions for preparing a Process Control solution can be found in the **Reagents Recipes** section of this procedure.

100% Recovery Standard:
In addition to adding Process Control spheres to each tissue and blood sample prior to processing, three new empty centrifuge tubes should also be prepared with only Process Control microspheres (no tissue).

Set these three tubes aside, as they will not go through any processing and digestion steps. These tubes will be poured onto three separate filters and then processed like tissue or blood samples as the 100% recovery standard. The results from these three tubes will be entered as Process Controls #1, #2 and #3 Column A of the **Data Calculation Page** of the Triton Technology Excel spreadsheet macros using the Process Control procedure. The software macro will calculate the average of these three readings as the 100% Recovery Standard value.

The Triton Technology Excel spreadsheets uses this average value to establish the 100% recovery value for the Process Control microspheres in each of the tissue and blood sample tubes.

TISSUE PROCESSING – FILTRATION

1.1 TISSUE SAMPLE

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 about 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 as the samples may have a bad smell. This will not affect the microsphere results. The samples should not be exposed to strong light as this may affect the microsphere dyes.

Alternatively, the tissue samples may be fixed with formalin. Formalin fixing will make the tissue digestion more difficult. 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 an alternate tissue fixing agent.

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 significant loss of spheres during tissue processing when using previously used centrifuge tubes.*

Screw-top Glass centrifuge tubes can also be used for processing of the tissue and blood samples. Glass tubes may be used over and over as long as they are cleaned.

Polystyrene centrifuge tubes should not be used, as polystyrene tubes are quite brittle and they may crack during the processing procedures.

Eye protection plus nitrile/latex gloves and should be used for all the sample processing steps, particularly those steps involving the ***Alkaline Tissue Digestion Reagent (ATDR)****. The KOH digestion reagent is very caustic and will burn exposed skin.*

1. **Tissue Sample Preparation:**
   Small tissue samples can be processed in 15ml 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 cleaner samples is that they are less likely to clog the filter during microsphere recovery.

   The 'wet weight' of each sample should be determined when the tissue samples are placed in their respective sample tubes. The 'wet weight' of a tissue sample is the combined weight of the tube and the tissue sample minus the empty ('tare') weight of the tube. The 'wet weight' of each sample is used in the computation of volume blood flow (ml/minute/gram).

   As the tissue samples are harvested they should be placed in centrifuge tubes and identified with a unique identification number or code. Use a permanent marker to identify each sample tube with the sample number. Tare the sample tube on an accurate scale. Always weigh the centrifuge tubes with their caps removed to increase the accuracy of the measurement. Place the tissue sample into the tube. Push the tissue sample to the bottom of its tube with a glass rod or briefly centrifuge each sample in order to force the tissue sample to the bottom of the tube. Re-weigh the tube plus sample and record the sample weight. Record the wet weight of the tissue sample along with the respective sample identification and description.

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

2. **Process Control Step** (strongly recommended)
   Use a repeating pipette to add Blue Process Control microspheres (typically 10,000 microspheres in 100μL) from a constantly-stirred beaker of Process Control solution to each tissue or blood sample tube prior to the following processing steps. The Blue microsphere Process Control suspension is made by diluting the factory concentration (3 million per ml) with the 0.05% Tween 80 solution per the instructions in the recipe section in the back of this procedure.

3. **Alkaline Tissue Digestion Reagent (4M ATDR)**:
   Nitrile or latex gloves and eye protection should be used for all the sample processing steps, particularly those steps involving the ***Alkaline Tissue Digestion Reagent (ATDR)****. The KOH digestion reagent is very caustic and will burn exposed skin. Eye protection should always be used when working with KOH reagents.*

   A. **15ml Sample Tube** (sample size of less than 3grams)**:**
   When using a 15ml centrifuge tube, add 12ml of ***4 Molar Alkaline Tissue Digestion Reagent*** **(4M ATDR)**. Place the screw cap on each tube and tighten snugly.

   NOTE: Do not use more than ***12ml*** *of* ***4 Molar KOH*** *in the 15ml tubes for ease of vortexing and in order to leave room for gas to build up during digestion.*

   B. **50ml Sample Tube** (larger size sample):
   When using a 50ml centrifuge tube, add 40ml of ***4 Molar Alkaline Tissue Digestion Reagent*** **(4M ATDR)**. Place the screw cap on each tube and tighten snugly.

   Do not use more than 40ml of ***4 Molar KOH*** *in the 50ml tubes for ease of vortexing and in order to leave room for gas to build up during digestion.*

4. Next go to **Section 1.3** of the Procedure, **OVERNIGHT ALKALINE HYDROLYSIS.**

1.2 REFERENCE BLOOD SAMPLE

Clotted blood is more difficult to digest. The reference withdrawal blood should be anti-coagulated with EDTA (1.5mg/ml of blood) as soon as it is withdrawn. Mix the EDTA into the blood in a capped tube by repeated inversion. Heparin or Citrate may also be used as a blood anti-coagulant.

*All reference blood samples should be processed in n**ew 50ml** disposable polypropylene centrifuge tubes in order to minimize the potential for microsphere loss during* processing.

Reference Blood Samples larger than 10ml must be split and processed in multiple 50ml centrifuge tubes.

Screw-top Glass centrifuge tubes can also be used for processing of the tissue and blood samples. Glass tubes can be re-used if washed well.

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

Eye protection plus nitrile/latex gloves and should be used for all the sample processing steps, particularly those steps involving the KOH digestion reagents.. The KOH digestion reagent is very caustic and will burn exposed skin.

1. **Process Control Step** (strongly recommended)
   Use a repeating pipette to add Blue Process Control microspheres (typically 10,000 microspheres in 100µl) from a constantly-stirred beaker of Blue Process Control solution to the entire blood sample prior to the splitting the blood sample in the following steps. Mix the blood sample well by vortexing or successive inversion prior to splitting the blood sample in the steps below. Split the blood sample into several new 50ml tubes as required for digestion per the directions below. The digested blood samples will be re-combined for dye analysis and Process Control determination.

2. **Alkaline Blood Digestion Reagent - 16M KOH (ABDR)**
   Each 10ml or less blood sample tube should be diluted up to the 30ml level with 2% Tween-80 reagent and vortex mixed. Add 10ml **16M KOH *Alkaline Blood Digestion Reagent* (16M ABDR)** to each reference blood sampl**e**, then vortex mix.

   The 50ml tubes should not be filled above the 40ml line for ease of vortexing and in order to leave room for gas to build up during digestion.

3. Next go to **Section 1.3** of the Procedure, **OVERNIGHT ALKALINE HYDROLYSIS**

1.3 OVERNIGHT ALKALINE HYDROLYSIS

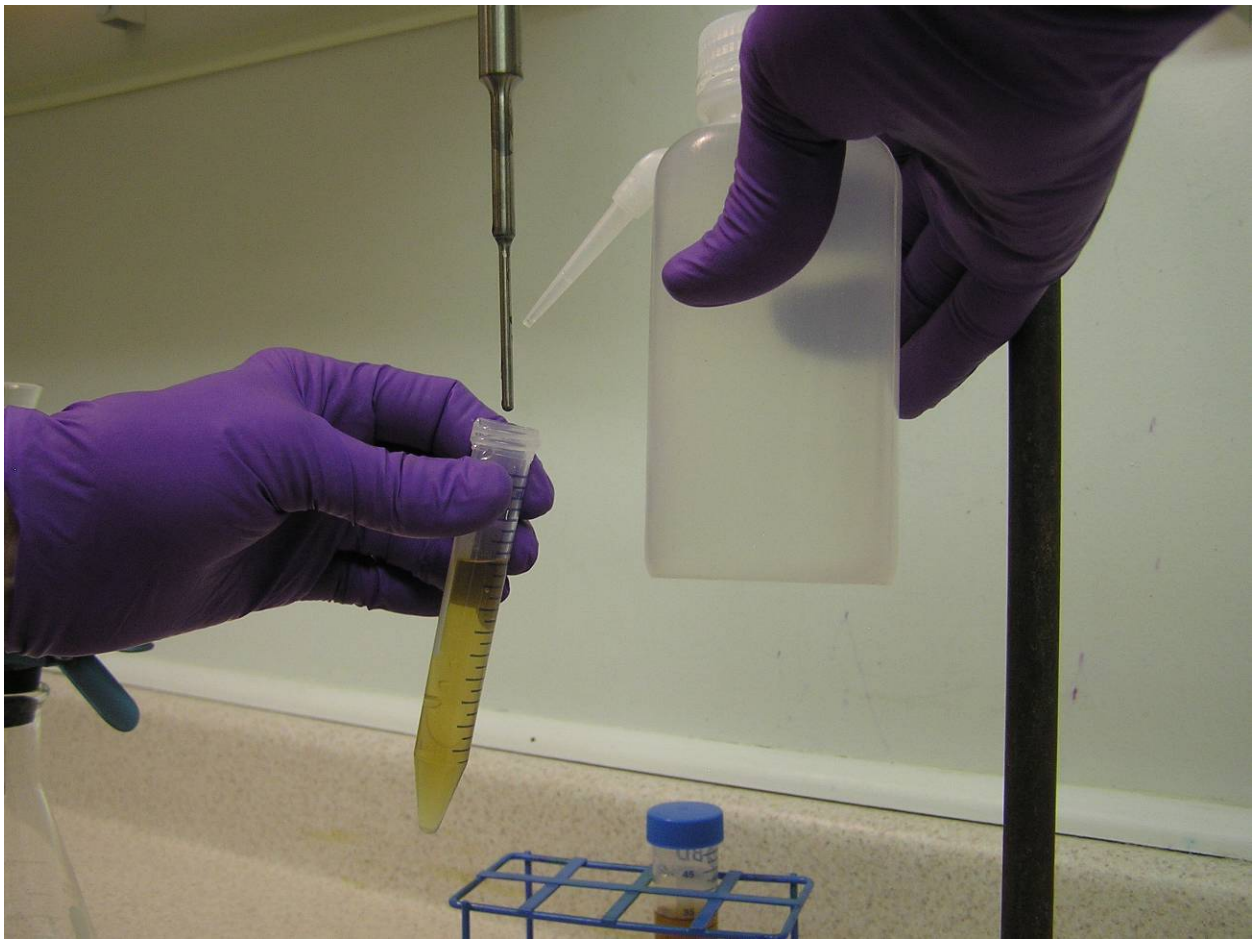
The use of nitrile or latex gloves is strongly recommended for all the sample processing steps, particularly those steps involving the ***KOH Alkaline Digestion Reagent****. The KOH digestion reagent is very caustic and will burn exposed skin. Eye protection should always be used when working with the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

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 processed 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 homogenizer (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 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 into the sample tube so that no microspheres are lost.

Typical Ultrasonic 'Sonicator' drive unit connects to an electronic control unit.

If sonicatior is not available, aggressive vortexing will be required to re-suspend the tissue and microsphere pellet in the following digestion steps.



Use a wash bottle filled with distilled water to rinse the sonicator probe tip back into the sample tube so that no microspheres are lost in the sonication step.

1. Place the tubes from the tissue and blood preparation steps in a temperature-controlled laboratory oven set to a maximum 50°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 re-warmed in the 50°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

Each day when a dye analysis session is to be done, the spectrophotometer should have a baseline calibration with a cuvette filed with the appropriate solvent. The baseline calibration should be performed by doing a 'step-wise' absorbance scan with a cuvette filled only with the appropriate solvent. The scan can be across the entire wavelength spectrum of interest, typically 350 to 700nm. Alternately, it is really only necessary to do the baseline scan at the wavelengths corresponding to the microsphere colors used in the experiment. The Triton blood flow analysis software will only use those specific wavelengths. Refer to the manual for your spectrophotometer to understand how to do a baseline calibration scan.

Good lab technique is always important. The cuvettes should always be clean inside and out between measurements, 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.*

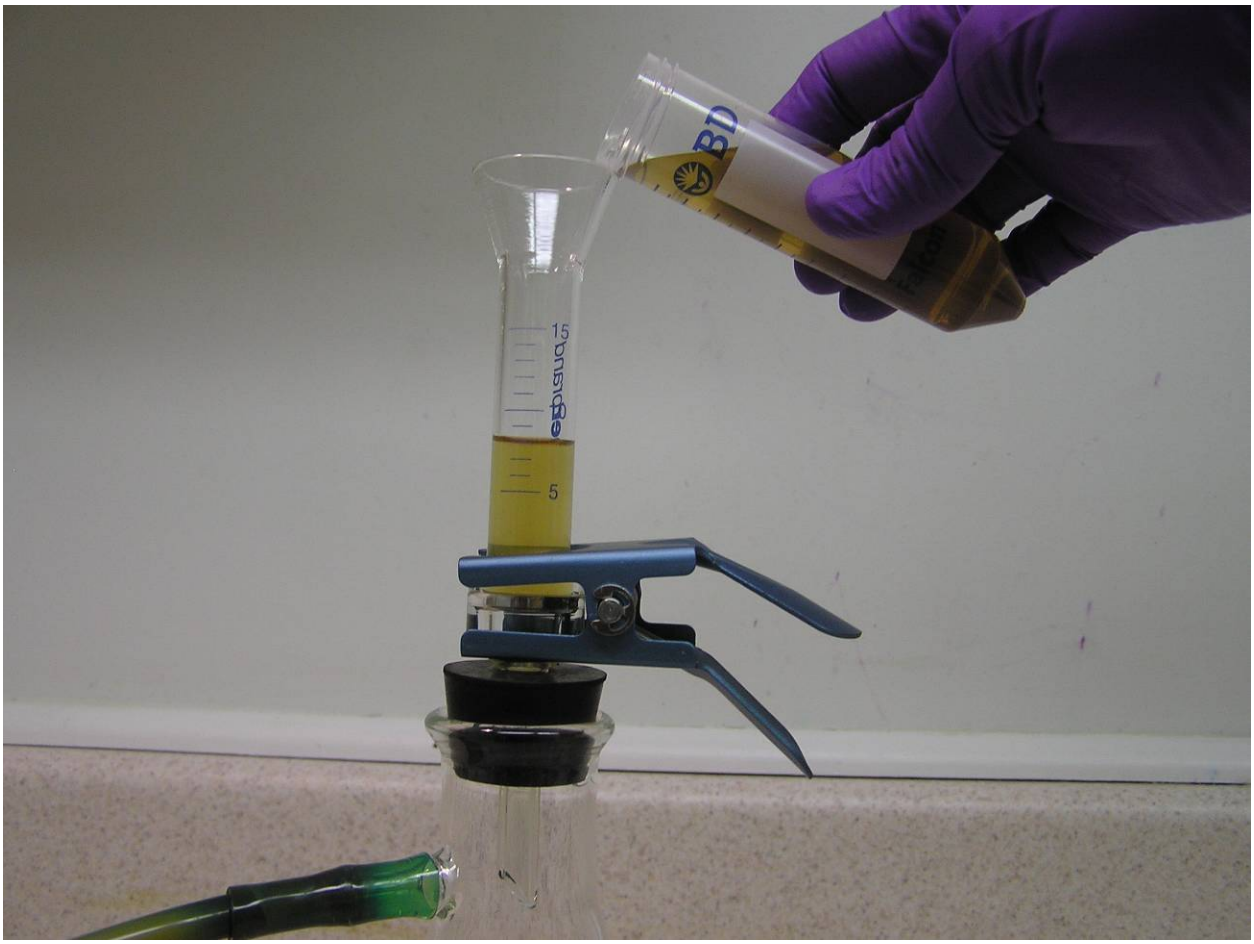
1. Assemble the filtration apparatus using a 25mm diameter 10µm pore size filter (Triton Technology Part #31079) between the upper graduated cylinder and the filter screen per the manufacturer's directions. Take care to insure that the filter is centered in the apparatus to prevent any unnecessary loss 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 to wet the filter. Then turn the vacuum source off.
2. Remove an individual tissue or blood samples from the 50°C oven, vortex mix the sample and pour all the contents of the sample into the graduated cylinder with the vacuum turned off. Pouring all the digested tissue (or blood sample) solution into the filter cylinder while vacuum is turned off gives a much more even distribution of the microspheres and tissue debris on the filter membrane. (See photo below)

   Working with a warm sample helps by keeping the fatty material in solution for ease of filtering. After the sample is in the cylinder above the filter membrane, turn the vacuum source on. When the contents of the cylinder have completely passed through the filter, turn the vacuum source off.

   Note: It is very helpful to have a foot-switch control for the vacuum source.
3. Rinse the sample tube with approx.10ml of 50°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 any microspheres that might remain on the walls of the sample tube. Hot water is used to keep any lipids and fats in solution and also helps prevent the filter from clogging. Turn the vacuum source on again to complete the filtering. When the filter is dry, rinse the walls of the filtration graduated cylinder with more heated distilled water to help dislodge any remaining microspheres. 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 the tube and then pour the contents into the graduated filter cylinder. An ETOH rinse will collapse any water-based bubbles on the walls of the centrifuge tube and thus help recover any microspheres remaining on the cylinder walls. 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. Start the 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 any residual KOH will have been minimized.

Typical vacuum filter apparatus for 25mm diameter filters

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 again and with the vacuum 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 similar tool. Handle the filter only by the edge where there will be no microspheres. Keep the filter level with the 'microspheres side' up. Carefully form the filter into a U-shape and insert the filter into the mouth of a 15ml conical centrifuge tube while holding the sample tube horizontal. Always keep the 'microsphere side' of the filter facing upwards. With care not to disturb the microspheres on the filter upper surface, push the filter to the bottom of the sample tube using a small spatula or similar tool.



Pour digested sample into filter apparatus, and then turn on vacuum.

9. Hold the centrifuge tube vertically and add 300µl of the appropriate solvent, if you are using a 200µl cuvette measurement. Use **DMF** for *original* 5-color Dye-Trak. Use ONLY ***acidified*** **Cellosolve Acetate** solvent for **Dye-Trak VII+**.

   Note: Failure to use the ***acidified Cellosolve Acetate*** *with Dye-Trak VII+ may cause variable results with some colors.*

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 (100µl to 200µl). Carefully extract the dye solvent sample from the sample tube using an adjustable pipette set for 200µL. It is important to extract the analysis *highest reading to* <u>*below 1.3AU*</u>. *This must be done in order to keep the reading in the linear re* 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

Using tweezers, carefully insert the partially folded filter into a 15ml conical tube

13. Pour the dye sample contents from the cuvette into a clean 15ml tube. Calculate the minimum amount of solvent that can be added to the dye solution to keep the peak reading below 1.3AU. Add that accurately measured volume of solvent to the 15ml tube and vortex mix. Do not over dilute the sample, as this will decrease the accuracy. Clean and dry the cuvette and return the diluted dye sampler to the cuvette for a second analysis. It is extremely important to keep track of any sample dilutions for the later calculations of blood flow. Record each extraordinary sample dilution for entry into the Excel spread sheet for that particular sample.
14. The spectrographic data for each tissue and blood sample should be recorded. Most contemporary spectrophotometers will generate an ASCII (or similar) data file for file storage. It is not necessary to do a complete spectral scan, it is only necessary to read the samples at the specific wavelength for each color of microsphere used in the experiment. Reading just the specific required wavelengths makes for smaller data files.
15. If you are using the Triton Technology Microsoft Excel analysis software macros, it is important that the samples are read at the specific wavelengths the software is looking to read. The Triton software is looking for wavelengths that are in 5nm increments. If you are going to do a wide spectrum scan, the spectrophotometer should be set to scan in either 1nm steps or 5nm steps. If 2nm steps are used the software macro will not read the data and give an error response.

Note: If the absorbance reading for any of the dye samples exceeds 1.3AU, then it will be necessary to dilute the sample with a measured volume of solvent sufficient to bring the absorbance readings into the linear region.

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 Homogenizer ('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 should be 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 conical test tube (approx. 2mm diameter).

   An ultrasonic cleaning bath will not work as a substitute; the sound waves do not effectively penetrate the plastic walls of the sample tubes.
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.
4. 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 storage media)

100µl to 200µl UV/VIS spectrophotometer microcuvette for spectrophotometer

Ring-Stand with clamps, bars, etc.

Power Syringe Withdrawal Pump (Kent Scientific)

200µl Adjustable Pipetter with disposable tips

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

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

Vacuum source, preferably connected to a foot control switch.

Pasteur pipettes with rubber suction bulbs

Filter Apparatus (Fisher Scientific)

Triton Technology Inc 25mm 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 homogenizer 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.)

1.6 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. Sodium Chloride should be USP grade. The numbers listed are representative only and other venders equivalent items can be used instead.

ETOH - Denatured Ethyl Alcohol, 1 Liter .....**Sigma-Aldrich** 493546

Tween 80, 250ml.....**Sigma-Aldrich** P1754

Triton X-100, 1 Liter.....**Sigma-Aldrich** X100

Potassium Hydroxide, pellets, (FW 56.11, 1kg).....**Sigma-Aldrich** 22147-3

Hydrochloric Acid, 37%, 500ml.....**Sigma-Aldrich** 25814-8

Sodium Azide*, (FW 65.01), 5 gram.....**Sigma-Aldrich** 438456

Thimerosal, (FW 404.8), 1 gram.....**Sigma-Aldrich** T8784
(Ethylmercurithiosalicylic Acid, sodium salt)

Sodium Chloride, USP(FW 58.44), 100 gram.....**Sigma-Aldrich** S1679

DMF** *N,N*-Dimethylformide, *spectrographic grade*, 1 Liter….**Sigma-Aldrich** 227056

Cellosolve Acetate** 2-Ethoxyethyl Acetate, 1 Liter.....**Sigma-Aldrich** 10996-7

* 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

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 (4M ATDR):
Add 1000 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 224.4gram 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 Tissue Digestion Reagent (4M KOH)".** *Caution: This is a very caustic solution. Wear Nitrile or Latex gloves and eye protection. Handle with extreme care!*

16M KOH Alkaline Blood Digestion Reagent (16M ABDR):
Add 1000 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 Nitrile or Latex gloves and eye protection. Handle with extreme care!*

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

10% Tween 80 Solution:
Add 90ml of distilled water to a 250ml 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 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. Add 10 milligrams Thimerosal to the solution if this solution is not going to be used immediately. If the reagent is going to be kept for a couple of weeks, the Thimerosal should be added and the reagent should be kept in a refrigerator. Store the solution in plastic bottles identified as **"10% Tween 80".**

0.05% Tween 80 + Saline Solution (Microsphere Carrier Solution):
Add 0.50ml **10% Tween® 80** solution to 99.5ml sterile saline solution in a 250ml beaker and mix by stirring. Add 10milligrams Thimerosal to the solution if it is not going to be used right away. If the reagent is going to be kept for a couple of weeks, then Thimerosal should be added and the reagent should be kept in a refrigerator. Make the reagent only as needed for use as an injectate solution. Store in plastic bottles identified as **"Saline plus 0.05%Tween 80 (MCS)".**

Process Control Solution:
Make up a solution with a concentration of 100,000 Blue spheres per using the **0.05% Tween 80 *Microsphere Carrier Solution* (MCS)** to dilute the factory concentration of 3 million microspheres per ml by a factor of 300. Use a 100µl 'repeating-pipette' to add **Process Control** spheres to each tissue/reference processing tube prior to hydrolysis. <u>The **Process Control** solution should be continuously stirred with a magnetic stir-bar while filling the 100µL repeating pipette to insure that each **Process Control** bolus contains the same number of spheres.</u>

H. TISSUE PROCESSING - SEDIMENTATION

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**

*Note: *We recommend that White be read at 390nm, rather than its peak absorbance (370nm) to minimize biologic UV baseline background noise. If your tissue samples do not contain high amounts of UV background absorbance, you might want to read White at the peak absorbance wavelength of 370nm. The Triton Technology data processing Excel macros will need to be modified to read White at 370nm*

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

Number of Colors Used	Colors to Use	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 its 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 by the Triton supplied Excel blood flow analysis software to correct the measured results for each tissue or blood sample.

Process Control *microspheres cost a few cents per tissue sample!*

If the amount of Blue 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 to the value that would have been measured had there been no loss. The free Triton Technology Excel volume blood flow calculation macros will detect and correct for any of microspheres for all the readings in an given sample.

Process Control is accomplished by using a repeating pipette to add a fixed number of Blue microspheres (typically 10,000), to each sample tube prior to the tissue digestion steps for each of the tissue and blood sample tubes. This can be done by adding 100µl of the Blue Process Control solution prepared using the recipe in the reagent section of this manual. The instructions for preparing the Process Control solution can be found in the **Reagents Recipes** section of this procedure. The Process Control solution should be constantly stirred with a magnetic stir bar while it is being used.

Blue microspheres are used for Process Control because the Blue color has the longest wavelength of all the microsphere colors and none of the other colors spill-over into the Blue absorbance peak.

100% Recovery Standard:
In addition to adding Process Control spheres to each tissue and blood sample prior to processing, three new empty centrifuge tubes should also be prepared with only Process Control microspheres (no tissue).

Set these three tubes aside, as they will not go through any processing and digestion steps. These three tubes will be processed like tissue or blood samples as the 100% recovery standard. This will involve partially filling the tubes with ETOH, vortex mixing, spinning down, aspirating down to a safe level, and then allowing the tubes to evaporate dry overnight. A known volume of solvent will be added to the tubes, just like tissue or blood samples and the solvent dye content analyzed. The results from these three tubes will be entered as Process Controls #1, #2 and #3 Column A of the **Data Calculation Page** of the Triton Technology Excel spreadsheet macros using the Process Control procedure. The software macro will calculate the average of these three readings as the 100% Recovery Standard value.

The Triton Technology Excel spreadsheets uses this average value to establish the 100% recovery value for the Process Control microspheres in each of the tissue and blood sample tubes.

TISSUE PROCESSING – SEDIMENTATION

1.1 TISSUE SAMPLE

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 about 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 as the samples may have a bad smell. This will not affect the microsphere results. The samples should not be exposed to strong light as this may affect the microsphere dyes.

Alternatively, the tissue samples may be fixed with formalin. Formalin fixing will make the tissue digestion more difficult. 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 will be any digestion or recovery problems. Spectrophotometric analysis must also be evaluated to confirm dye absorbance profile and intensity stability before using an alternate tissue fixing agent.

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 significant loss of spheres during tissue processing when using previously used centrifuge tubes.*

Screw-top Glass centrifuge tubes can also be used for processing of the tissue and blood samples. Glass tubes may be used over and over as long as they are cleaned.

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

Eye protection plus nitrile/latex gloves and should be used for all the sample processing steps, particularly those steps involving the ***Alkaline Tissue Digestion Reagent (ATDR)****. The KOH digestion reagent is very caustic and will burn exposed skin.*

1. **Tissue Sample Preparation:**
   Small tissue samples can be processed in 15ml 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 cleaner samples is that they are less likely to clog the filter during microsphere recovery.

   The 'wet weight' of each sample should be determined when the tissue samples are placed in their respective sample tubes. The 'wet weight' of a tissue sample is the combined weight of the tube and the tissue sample minus the empty ('tare') weight of the tube. The 'wet weight' of each sample is used in the computation of volume blood flow (ml/minute/gram).

   As the tissue samples are harvested they should be placed in centrifuge tubes and identified with a unique identification number or code. Use a permanent marker to identify each sample tube with the sample number. Tare the sample tube on an accurate scale. Always weigh the centrifuge tubes with their caps removed to increase the accuracy of the measurement. Place the tissue sample into the tube. Push the tissue sample to the bottom of its tube with a glass rod or briefly centrifuge each sample in order to force the tissue sample to the bottom of the tube. Re-weigh the tube plus sample and record the sample weight. Record the wet weight of the tissue sample along with the respective sample identification and description.

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

2. **Process Control Step** (strongly recommended)
   Use a repeating pipette to add Blue Process Control microspheres (typically 10,000 microspheres in 100µl) from a constantly-stirred beaker of Process Control solution to each Tissue or Blood Sample tube prior to the following processing steps. The Blue microsphere Process Control suspension is made by diluting the factory concentration (3 million per ml) with the 0.05% Tween 80 solution per the instructions in the recipe section.

3. **Alkaline Tissue Digestion Reagent (1M ADR)**:
   *Nitrile or latex gloves and eye protection should be used for all the sample processing steps, particularly those steps involving the **1M Alkaline Tissue Digestion Reagent (1M ADR)**. The KOH digestion reagent is very caustic and will burn exposed skin.*

   *It is very important not to use a KOH solution more concentrated than 1 molar when using the **Sedimentation** process. The increased density of higher molarity KOH 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.*

   A. **15ml Sample Tube** (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.

   *Do not use more than **6ml** of **1 Molar KOH** in the 15ml tubes for ease of vortexing and in order to leave room for gas to build up during digestion.*

   B. **50ml Sample Tube** (larger size sample):
   When using a 50ml centrifuge tube, add 2ml0 of ***1 Molar Alkaline Digestion Reagent*** **(1M ADR)**. Place the screw cap on each tube and tighten snugly.

   *Do not use more than **20ml** of **1 Molar KOH** in the 50ml tubes for ease of vortexing and in order to leave room for gas to build up during digestion.*

4. Next go to **Section 1.3** of the Procedure, **OVERNIGHT ALKALINE HYDROLYSIS.**

1.2 BLOOD SAMPLE

Coagulated blood is difficult to digest than blood that has been anti-coagulated. The preferred course of action is to add Blood Hemolysis Reagent (BHR) to the Reference Blood Samples and mix by repeated inversion in a capped tube as soon as the blood is 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 the EDTA into the blood in a capped tube by repeated inversion. Heparin or Citrate may also be used as a blood anti-coagulant.

All reference blood samples should be processed in ***new 50ml*** *disposable polypropylene centrifuge tubes in order to minimize the potential for microsphere loss during* processing.

Reference Blood Samples larger than 20ml must be split and processed in multiple 5ml centrifuge tubes.

Screw-top Glass centrifuge tubes can also be used for processing of the tissue and blood samples. Glass tubes can be re-used if washed well.

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

Eye protection plus nitrile/latex gloves and should be used for all the sample processing steps, particularly those steps involving the ***Alkaline Digestion Reagent (ADR)****. The KOH digestion reagent is very caustic and will burn exposed skin.*

1. **Process Control Step** (strongly recommended)
   Use a repeating pipette to add Blue Process Control microspheres (typically 10,000 microspheres in 100µl) from a constantly-stirred beaker of Blue Process Control solution to the entire blood sample prior to the splitting the blood sample in the following steps. Mix the blood sample well by vortexing or successive inversion prior to splitting the blood sample in the steps below. Split the blood sample into several new 50ml tubes as required for digestion per the directions below. The digested blood samples will be re-combined for dye analysis and Process Control determination.
2. 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. This step must be done prior to adding the KOH or the blood will clump in KOH.
3. Centrifuge the tubes containing the hemolized blood for at least 15 min. at 1,500g , then aspirate the supernate solution down to a level safely above visible pellet. The pellet may not be easy to see, so when in doubt, aspirate *no lower than the 10ml volume mark*.
4. **Alkaline Digestion Reagent (1M ADR):**
   It is important *not* to use a KOH solution more concentrated than 1 molar when using the **Sedimentation** process. The increased density of higher molarity KOH 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 50ml Reference Blood centrifuge tube. 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 !
5. Next go to **Section 1.3** of the Procedure, **OVERNIGHT ALKALINE HYDROLYSIS**.

1.3 OVERNIGHT ALKALINE HYDROLYSIS

Eye protection should always be used when working with the KOH reagent. Nitrile or latex gloves are recommended for all the sample processing steps, particularly those steps involving the ***KOH Alkaline Digestion Reagent****. The KOH digestion reagent is very caustic and will burn exposed skin.*

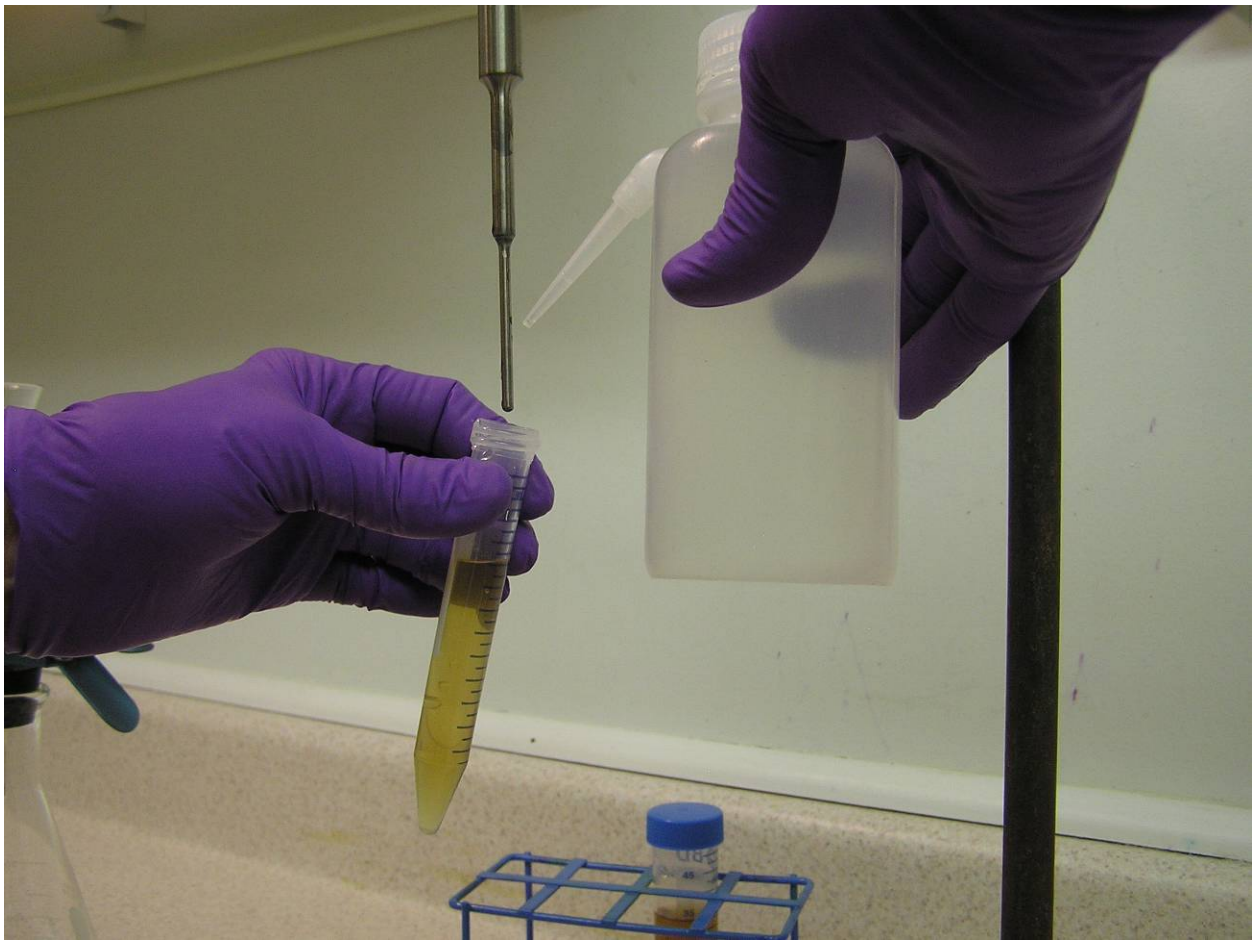
It is assumed that a number of tissue / blood samples will be processed at the same time. The number of tubes that can be held in the centrifuge will limit the number of tubes that are processed at one time.

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 homogenizer (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 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 into the sample tube so that no microspheres are lost

Typical Ultrasonic 'Sonicator' drive unit connects to an electronic control unit.

If sonicatior is not available, 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 50°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.



Use a wash bottle filled with distilled water to rinse the sonicator probe tip back into the sample tube so that no microspheres are lost in the sonication step.

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 for any reason samples are allowed to cool, they should be re-warmed in the 50°C oven prior to the microsphere recovery step.

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 sonicoator 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).*
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 with a cuvette filed with the appropriate solvent. The baseline calibration should be performed by doing a 'step-wise' absorbance scan with a cuvette filled only with the appropriate solvent. The scan can be across the entire wavelength spectrum of interest, typically 350 to 700nm. Alternately, it is really only necessary to do the baseline scan at the wavelengths corresponding to the microsphere colors used in the experiment. The Triton blood flow analysis software will only use those specific wavelengths. Refer to the manual for your spectrophotometer to understand how to do a baseline calibration scan.

Good lab technique is always important. The cuvettes should always be clean inside and out between measurements, 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 150µl 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 200 µl 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 Pasture 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.

4. Transfer the supernate from the sample tube to the appropriate sized microcuvette (100 µl to 200 µl). If any of the absorbance-peaks in a sample exceed 1.3AU 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 its '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.

1.5 HINTS AND NOTES

1. Tissue from the gastrointestinal (GI) track, even when less than 3 grams, should be processed in the larger 50 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 Homogenizer ('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 should be 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 conical test tube (approx. 2mm diameter).

   An ultrasonic cleaning bath will not work as a substitute; the sound waves do not effectively penetrate the plastic walls of the sample tubes.
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.
4. 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 storage media)

100 µl to 200 µl UV/VIS spectrophotometer microcuvette for spectrophotometer

Ring-Stand with clamps, bars, etc.

Power Syringe Withdrawal Pump (Kent Scientific)

200µl Adjustable Pipetter with disposable tips

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

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

Vacuum source, preferably connected to a foot control switch.

Pasteur pipettes with rubber suction bulbs

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 homogenizer 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. Sodium Chloride should be USP grade. The numbers listed are representative only and other venders equivalent items can be used instead.

ETOH - Denatured Ethyl Alcohol, 1 Liter .....**Sigma-Aldrich** 493546

Tween 80, 250ml..... .....**Sigma-Aldrich** P1754

Triton X-100, 1 Liter.....**Sigma-Aldrich** X100

Potassium Hydroxide, pellets, (FW 56.11, 1kg).....**Sigma-Aldrich** 22147-3

Hydrochloric Acid, 37%, 500ml.....**Sigma-Aldrich** 25814-8

Sodium Azide*, (FW 65.01), 5 gram..... .....**Sigma-Aldrich** 438456

Thimerosal, (FW 404.8), 1 gram..... ...**Sigma-Aldrich** T8784
(Ethylmercurithiosalicylic Acid, sodium salt)

Sodium Chloride, USP(FW 58.44), 100 gram.....**Sigma-Aldrich** S1679

DMF** *N,N*-Dimethylformide, *spectrographic grade*, 1 Liter.....**Sigma-Aldrich** 227056

Cellosolve Acetate** 2-Ethoxyethyl Acetate, 1 Liter.....**Sigma-Aldrich** 10996-7

* 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

Note: All reagents can be stored at room temperature:

Alkaline Digestion Reagent (1M ADR):
Add 2000 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 eye Latex gloves and handle with care!**

Acidified Ethanol Reagent:
Prepare a stock solution of *acidified ethanol* by adding hydrochloric acid (HCl, 37%/10N) 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 10µl hydrochloric acid (HCl, 37%/10 Normal) to 100ml of Cellosolve Acetate**.** This bottle should be identified as **"Acidified 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 0.20gram 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 300 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".**

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 0.05gram 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)".**

Blood Hemolysis Reagent:
Add 1 liter of 10% Triton X-100 solution 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)".**

Process Control Solution:
Make up a solution with a concentration of 100,000 Blue spheres per using the **0.05% Tween 80** ***Microsphere Carrier Solution*** **(MCS)** to dilute the factory concentration of 3 million microspheres per ml by a factor of 300. 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 with a magnetic stir-bar while filling the 100µL repeating pipette to insure that each **Process Control** bolus contains the same number of spheres.

APPENDIX A - Material Safety Data Sheet

I. Product Identification

PRODUCT: Colored Particle Size Standards

CONTENTS: Aqueous Suspensions of Polymer Microspheres containing trace amounts of colored Organic Dye. Polymers include: Polystyrene, Polystyrene Divinylbenzene, and Polymethylstyrene.

II. Hazardous Ingredients

Typical Values:

Ingredients	Percent	Hazard Data
Solid Plastic Spheres	0.05-2.0	Non-hazardous
Water	89-99.95	Non-hazardous
Polyoxyethylenesorbitan	0.05	Non-hazardous
Thimerosal	0.01	Non-hazardous
Organic Dye	<0.005	Non-hazardous

III. Physical Data

Boiling Point:	100 C
Vapor Density:	NA
Volatiles, percent by volume:	89-99.95% as water
Water Solubility:	Material is a suspension of insoluble colored plastic microspheres in water.
Specific Gravity:	1.05-1.09
Molecular Weight:	Varies
Appearance:	Colored liquid when shaken, otherwise clear with colored layer on bottom

IV. Health Hazard Data

EFFECTS OF EXPOSURE:

Eyes: Possible minor irritation.

Skin: Non-irritating, except for prolonged and repeated exposure.

Ingestion: Possible minor gastric irritation.

Inhalation: No known adverse effects, but respiration of fine particles should be avoided as a general principle.

FIRST AID:

Eye Contact: Wash thoroughly with water. If irritation persists, consult a physician and stain for corneal abrasion.

Skin Contact: Wash thoroughly with soap and water.

Inhalation of dry particles: Remove to clean air. Consult a physician if irritation persists.

Ingestion: For large volumes, induce vomiting and consult a physician.

V. Fire and Explosion Hazard Information

The suspensions are non-flammable. For dried material, use CO2, water or dry chemical extinguisher. Combustion of dry particles may produce noxious gases. Use suitable breathing equipment.

VI. Reactivity Data

Stability: The suspensions are chemically stable and no polymerizations will occur. They are incompatible with highly ionic solutions; such solutions will cause the particles to flocculate.

VII. Storage and Handling Precautions

Avoid creating, ingesting or inhaling dusts or aerosols of fine particles. Keep tightly sealed to prevent contamination. Avoid damaging or puncturing containers. Store between 4C and 25 C. DO NOT FREEZE.

VIII. Special Protection Information

Respiratory Protection: None required under normal usage. Filtered respirator is recommended for aerosol or dust production.
Ventilation: Normal ventilation is sufficient for most applications.
Gloves: Recommended.
Eye Protection: Highly recommended.
Protective clothing: Recommended.

IX. Spill, Leak and Disposal Procedures

Spills: AVOID CREATING, INGESTING OR INHALING DUSTS OR AEROSOLS. Wipe or wash up material. Caution: Surfaces covered with dried microspheres may become slippery.

Disposal: Small volumes (0.1 liter or less) may be disposed of in laboratory sinks and drains. Flush with water. Material may clog drains in large quantities. Bury large amounts of dried material in approved landfill.

APPENDIX B – EXCEL SPREADSHEET INSTRUCTIONS

Excel File Instructions and Notes for *original* Dye-Trak and Dye-Trak VII+

(version 0710)

(print with color printer for clarity)

OVERVIEW:

The Triton Technology spreadsheet macro is designed for use with both families of Dye-Trak absorbance-dyed microspheres. Using this spreadsheet macro for Microsoft Excel automates the process of entering spectrophotometer readings and, with some additional user supplied information, computes volume blood flow for each experimental tissue sample.

The first step is to enter the single-color standard curve data for each experimental color being used into the spreadsheet. The macro uses the single color spill-over data to perform a matrix inversion calculation for each measured sample, removing the spectral 'spill-over' between colors. This is identical to the matrix-inversion spill-over computation done with radioactive microsphere data.

There are several versions of the matrix inversion software. There is a separate version for each number of colors used in an experiment. This is done in order to minimize any 'noise' in the computations. In addition, there are also versions of the spreadsheet for use with a 'Process Control' color. The Blue (670nm) colored spheres are usually used for Process Control with Navy (650nm) colored spheres are an alternative. None of the other Dye-Trak colors spill-over into the Blue or Navy's absorbance peak. When the 'Process Control' is used, the spreadsheet macro will determine if any of microspheres in a sample have been lost during tissue processing and microsphere recovery. If any portion of the microspheres in a sample have been lost, the spreadsheet macro will determine the percentage of the sample that was lost. The recovery percentage for each sample is listed on the **RBF Calculation** page in column 'F'. The recovery value should ideally be close to 100%. The macro will then use the 'Process Control' data for each individual sample to correct the blood flow values for that sample to the values that would have been if there was no lossin the sample.

The spreadsheet file incorporates Visual Basic modules that will automatically open data files from various makes of spectrophotometer. These macro modules import the absorbance values at wavelengths defined in the **Data Calculation** page into the **FileDataPage** of the spreadsheet. The automatic data importation speeds up data entry and eliminates errors. The user can manually enter the required data, but this is time consuming and can result in entry errors.

After the Dye-Trak Excel program has been run and the Regional Blood Flow is computed for each sample on the **RBF Calculation** page. Once the calculations have been done the resultant file should be saved with a new name which refers to the particular experiment. This will save the data from the experiment and allow it to be accessed at any later time.

Copies of the original Excel '.xls' files should be kept in a separate directory so that they are not overwritten.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

EXCEL MACRO FILE NAMING STRUCTURE:

It is important to use the correct version to the Triton Technology Excel spreadsheet macro. Specific versions of the Excel spreadsheet macro are available for both the *original* Dye-Trak and the Dye-Trak VII+ family of colors.

There are versions for each number of experimental colors and it is important to use the correct version. If the macro software version for five colors is used for an experiment where only three colors were actually used, and 'zeros' forced in as data for the missing colors, the calculated blood flow values will be less accurate due to computational 'noise'.

Further, there are versions of the software for both Dye-Trak families with and without Process Control. We strongly recommend the use of Process Control to detect microspheres lost during the tissue/blood processing steps. Process Control corrects the measured AU values to what they would have been if no microsphere were lost during tissue processing. The use of Process Control increases the accuracy of the blood flow measurement at the additional cost of only a few cents per sample.

Listed below are examples of Excel macros file names for each of the Dye-Trak families. Each macro file is named in the following fashion:

TritonVII+ 6 Plus Control	Dye-Trak VII+ family of microspheres Six experimental colors plus a Process Control
TritonVII+ 6	Dye-Trak VII+ family of microspheres Six experimental colors (no Process Control)
Triton4 Plus Control	*original* Dye-Trak family of microspheres Four experimental colors plus a Process Control
Triton5	*original* Dye-Trak family of microspheres Five experimental colors (no control)

FILE ARCHIVING:
When running the Excel macro modules, the file should be saved with a new name after all the data is entered (absorbance values, tissue weights, blood withdrawal rates, etc). This new file name should be one that uniquely identifies the experiment.

For example**:** TritonVII+6 Plus Control.xls might be saved as **Experiment 3-1.xls** where this is the first animal in Experiment #3.

IMPORTANT: Backup copies of all the original Excel macro files should be saved in a separate archive directory.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

Spreadsheet Cell Color convention:
The data input areas are divided into the following general groups according to cell background color. Verify that all data input is in the correct units as specified by the topic header.

Blue = **keyboard data entry**
Type the correct information answering the topic expressed by a row or column header.

Yellow = **absorbance data entry**
The user can 'Copy and Paste' the absorbance values brought into the **FileDataPage** with the *'DyeTrak'* macro into the **Data Calculation** page. Alternatively, the user can manually type the absorbance data values from spectrophotometric measurement directly into the **Data Calculation** page. Carefully check manually entered data for typing errors.

White = **restricted regions**
These areas should not be overwritten or modified as they may contain crucial calculation formulas and paste-links. Modification of these areas can have unanticipated effects on other areas of the spreadsheet.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

SPREADSHEET PAGE OVERVIEW:

DATA CALCULATION

This is the 'top page' for the spreadsheet, where all the identifying information describing the experiment is entered (experiment name, date, etc.). On this page the 'single color' standard data for the matrix inversion calculations is entered, either manually or transferred from elsewhere utilizing the cut-and-paste entry. If Process Control is being used, the three Process Control data readings are also entered on this page. One Process Control tube is usually taken at the beginning of the sample processing, one in the middle of the run and one at the end of the sample processing run. The average value of these three Process Control readings is used to establish the '100% percent recovery value' for the tissue and blood samples (**Data Calculation page, cell E43**).

The 'Reference Withdrawal' blood data is also entered on the **Data Calculation** page. A powered syringe is used to withdraw blood a major artery during the injection of each microsphere color into the circulation. This blood sample determines the concentration of the microsphere in the circulating blood. An accurate representative value for the Reference Withdrawal rate must be entered, as this value directly effects the calculated blood flow for all samples (**Data Calculation page, cells E50, E51, etc**). Always double check manually entered absorbance values for accuracy.

Lastly, the descriptive data for each sample is entered on this page. This data will be a description of the sample, the 'wet weight' of the sample, the volume of solvent used to extract the dye from each sample and the unique sample identifying number that is on the sample tube. The absorbance values for each microsphere color associated with the sample can also be manually entered on each sample line if those values were not transferred by 'cut and paste' from the **FileDataPage**.. This will include the absorbance for each color wavelength used in the experiment. It is very important to keep track of the amount of solvent for each sample. If a sample requires additional dilution beyond the normal amount, this additional solvent must be taken into account to determine the actual blood flow value. The correct solvent volume should be entered for each sample.

If a Reference Withdrawal blood sample is going to be split for digestion, then the Process Control spheres should be added and the blood sample well mixed before the blood sample is split among several sample tubes. The blood sample will be recombined in the later steps of the blood processing and the Process Control spheres will correctly indicate any loss of spheres from any or all of the blood tubes. The final values for the combined blood sample will indicate the corrected absorbance values for the whole blood sample.

FileDataPage

The **FileDataPage** is where the spectral data for each experiment is input into the spreadsheet from the ASCII spectrophotometer files. Normally the data will be entered into this page automatically from the spectrophotometer data file using the appropriate macro for your spectrophotometer's data format. Once the relevant has been up-loaded into the **FileDataPage,** it can be 'cut-and-pasted' into the **Data Calculation** spectrophotometer data cells (upper left corner is cell E58).

For small experiments or when using a spectrophotometer that does not output ASCII data files, absorbance data can be entered manually directly into the **Data Calculation** page. Always double check manually entered values for accuracy.

RBF Calculation

On this page the true microsphere 'counts' are determined by removing the spill-over errors using the Single Color Standard curve information to perform the *matrix inversion* calculations. If Process Control is being utilized, the data is also corrected for sample loss during tissue processing and microsphere recovery. Regional Blood Flow (RBF) for each tissue sample the computed using the spill-over and Process Control corrected absorbance data and the results listed on this page.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

RBF Chart

This page graphically displays the Regional Blood Flow for each tissue sample computed on the **RBF Calculation** page.

DYE-TRAK ABS Data Scan Profiles

Typical spectral scans are shown for each of the colors used in this spreadsheet. These data points are taken at 2nm increments and this data is shown for informational use only. This date should not be used as the 'single color' standards on the **Data Calculation** page

NORMALIZED PEAK PROFILES

The normalized profile chart is shown for reference to inform the user of the typical spectral characteristics of the Dye-Trak colors used in this spreadsheet.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

PAGE DETAILS:

FileDataPage:

The **FileDataPage** is used to automate data transfer from spectrophotometer ASCII data files into the spreadsheet. The Excel macro will automatically open spectrophotometer ASCII data files, search for the required spectrophotometer wavelengths using the values listed on the **DataCalculation** page. The macro will then copy the corresponding absorbance values onto the **FileDataPage.**

It is critically important that the spectrophotometer read absorbance in 1nm or 5nm steps. If the absorbance values are read in any other increments the macro will fail to load and give and error message. It is not necessary to do a complete spectral scan. The software macro only reads the wavelength values specified in the spreadsheet.

The automatic importation of the spectrophotometer files is implemented by clicking on **'DyeTrak'** callout in the upper tool bar. **'DyeTrak**' is located at the right hand end of the tool bar, adjacent to **'Help'.**

There are several different importation macro options for many brands of spectrophotometer. Select your brand of spectrophotometer from the list. If your first selection does not work, try another option, as some manufacturer's offer several ASCII formats and the macros can often read other formats. If you try all the file transfer options and cannot find one that works, contact Triton Technology for help.

The software macro will next ask to be directed to the first data file that is to be transferred.

It is important that the experimental data files to be transferred follow a strict file naming format described below. If the file naming structure in not followed, the macro will fail and give an error message.

Follow the same file naming pattern for all the tissue files that are to be analyzed in one run. It may be necessary to rename the files from spectrophotometer and saved in your computer. The files must have acceptable names and file extension in order for the Excel macro to import them (see next section).

SPECTROGRAPHIC DATA FILE NAMING RULES:

1: File *name prefix* must be constant for all samples from a single experiment. All the files must have a consistent name such as:

Dog 1, or **Experiment 7**, or **Study 3-4**, etc.

2: File *name extension* must be a three character numeric, increasing sequentially from ".001", ".002", etc up to the last sample to be analyzed.

Example: **Experiment 7.001**,
Experiment 7.002,
Experiment 7.003,
Experiment 7.004,
……..

Example: **Dog 1.023** Analysis file from the 23rd sample of **Dog 1**

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

USING THE *DYE-TRAK* DATA TRANSFER MACRO:

1 Select the appropriate ASCII spectrographic file structure for your spectrophotometer from the '***DyeTrak***' drop-down tool bar menu options.

2 Navigate to the file directory containing the set of ASCII data files to be opened.

3 Select the first file from the series of like-named files, with ascending numbered file extensions. Usually this will be the data file containing the ".001" file extension. Then click "OPEN".

4 The Excel macro will open this file and transfer the absorbance values into the spreadsheet on the **FileDataPage** for the wavelengths specified on the **Data Calculation Page**.

The macro will continue to open all the files with the same file name and having the sequential extensions ".002", ".003", ".004", … and transfer data sequentially for every file into the **File Data Page**.

Caution: The macro will stop when it reaches the end of the sequence, or if it comes to a gap where a file does not exist for the next numeric extension in the sequence. In the following sequence the macro will stop importing after the fifth file, as the file for Sheep A.006 is missing.

Sheep A.001
Sheep A.002
Sheep A.003
Sheep A.004
Sheep A.005
Sheep A.007
Sheep A.008
Sheep A.009
Sheep A.010
Sheep A.011

Spectrophotometer Set-Up:
An execution error will occur if the wavelengths found within the ASCII data file do not match all of the wavelengths specified on **Data Calculation** page. To avoid this problem you should set the spectrophotometer to read in either 1nm or 5nm steps. It is only necessary that the spectrophotometer read the wavelengths listed on the **Data Calculation** page. If the spectrophotometer reads in 2nm steps the macro will fail when in cannot find the first odd numbered wavelength listed on the **Data Calculation** page**.**

If this occurs, re-open the Excel file and modify the wavelengths specified on the **Data Calculation** page to match those in the ASCII data file. If the spectrophotometer was accidently set to read in 2nm steps the data is not lost, as the file can be manually modified to the necessary wavelengths. The difference of 1nm will not affect the results.

For instance the reading at 544 or 546nm can be modified to indicate that the reading was made at 545nm, etc.

On the **'FileDataPage',** the column containing the header **'Sample Label'** allows the user to specify an experimental label for each ASCII file read.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

Below is an example of how the '**FileDataPage'** would appear following a limited file run and file labeling.

DATA CALCULATION PAGE:

The **Data Calculation** page is where both absorbance date and experimental parameter data are input into the spreadsheet for computation and display.

Characterization of Microsphere Spectra Profiles (Single Color Standards)

The Characterization of Microsphere Spectra Profiles table reflects the analysis of each individual microsphere color at each of the several color peak wavelengths used in the experiment. Each experiment performed should have new Microsphere Spectral Profile measurements for all the colors used in the experiment. This should be done routinely in case the response of the spectrophotometer has changed, or a new lot of microspheres is being used, etc. It is good laboratory practice to run new single color standards for each experiment. The Microsphere Spectra Profile defines the spectral overlap or spill-over between the various microsphere colors employed. This data is crucial to the accuracy of the matrix inversion calculation and therefore the accuracy of the blood flow results.

Experimental tissue samples measured will contain a composite absorbance spectrum reflecting all of the microsphere colors present. The measurement at a specific microsphere color peak wavelength will reflect both the quantity of that specific microsphere color, but also a spill-over contribution from the other microsphere colors. The spreadsheet macros use the Microsphere Spectra Profile data and the Excel matrix inversion routines to compute the measured absorbance at each wavelength to what it would have been if no spill-over between colors.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

Microsphere Spectra Profile preparation:

Each individual color of microsphere color must be analyzed alone to determine the spill-over into other colors peak wave length. This process should be done prior to each experiment. This data is used during the matrix inversion computation to remove the spill-over components.

1. Add 5 ml Ethanol to a 15 ml glass/polypropylene centrifuge tube

2. Re-suspend Dye-Trak microspheres in the original 20 ml bottle by vortex and vigorously inversion mixing.

3. Withdraw a 50 ul sample of the microsphere stock suspension. This sample will contain 150,000 microspheres.

4. Add the 50 ul microsphere sample to the ethanol solution. Rinse the pipette tip into the ethanol solvent by repeated filling and flushing to clear out any trapped spheres. Then vortex mix the ethanol / microsphere solution.

5. Centrifuge the solution at 1500g for 5 min. Carefully aspirate the supernatant down to a safe level above the microsphere pellet.

6. Allow the Ethanol to evaporate away and dry microspheres in a 50 deg C. in laboratory oven, or at room temperature overnight.

7. Add 5 mL solvent, either acidified Cellosolve Acetate or DMF, as appropriate for the Dye-Trak (See **Sedimentation** or **Filtration** tissue processing procedure for details). All samples within an experiment must employ the same solvent that is to be used in the experiment, as the solvent type affects the color spectrum.

8. Vortex mix, then let the mixture stand for 10 min to elute the dye completely.

9. Centrifuge at 1500g for 5 min.

10. Analyze each solution for absorbance at the each of the peak wavelengths employed in the experiment. The solution being analyzed is 150,000 microspheres in 5ml which is equivalent to 3,000 microspheres/100µl solvent.

11. Alternatively, one may scan the complete spectrum from 350 nm - 800 nm in 1 or 5 nm increments and save the results in an ASCII data file to be imported later.

12. Update the Microsphere Spectra Profile table with analysis results.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

Shown below is a partial example of the **Microsphere Spectra Profile** following analysis:

		Process Control Navy	Persimmon	Tangerine	Orange
		ABS Wavelength (nm)	ABS Wavelength (nm)	ABS Wavelength (nm)	ABS Wavelength (nm)
Input primary wavelength measured →		650	545	525	495
Process Control Navy	3,000	0.8760	0.0615	0.0218	0.0023
Persimmon	3,000	0.0000	1.2600	0.5719	0.2122
Tangerine	3,000	0.0000	0.2814	1.0980	0.3766
Orange	3,000	0.0000	0.0000	0.0000	1.0050
Yellow	3,000	0.0000	0.0000	0.0000	0.0000
Lemon	3,000	0.0000	0.0000	0.0000	0.0000
	Sphere number/100 uL ↑				

Cellosolve/DMF
Solvent Volume, uL
100
100
100

DMF or Acidified Cellosolve solvent Volume (ul)

The volume of solvent that is used in extracting the dye from within the microspheres. It is recommended to use 200 uL with most reference and tissue samples. If the peak absorbance is above 1.3 AU, the sample will need to be diluted with solvent to bring the peak AU into the linear absorbance range.

If the initial sample was extracted with 200 uL solvent and a subsequent 2-fold dilution was made with solvent to bring the peak absorbance down into the linear range, the recorded volume of solvent would be 400 uL.

Remember to record the dilutions when using other than your normal amount of solvent.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

Sample Calculation Table

The areas with a blue background will require user keyboard input of the appropriate value. The areas with a yellow background will require input of the appropriate absorbance measurement values. If the ***DyeTrak*** data transfer module has been used to transfer data values onto the **FileDataPage**, simply copy-and-paste the values from there into the appropriate fields of the **Data Calculation** page.

Process Control Microspheres:
Three samples define the number of Process Control microspheres added to each reference blood and tissue sample. Three empty tubes should be placed in the queue of tissue and blood tubes for analysis. These three tubes will only receive the pipetted Process Control microspheres. One empty tube should place at the start of the tissue processing run, the second tube placed in the middle of the run and the third empty tube should be placed at the end of the run. These tubes should be marked **Process Control 1**, **Process Control 2**, and **Process Control 3** with a water-proof label or marker. These three Process Control samples should be set aside as they will not go through the digestion procedure. The Process Control samples will be re-introduced to the sample queue when the tubes are at the final ethanol drying stages leading to evaporative drying, solvent dye extraction and analysis. Note that Blue (670nm) or Navy (650nm) can be used as the Process Control color, but Blue is the preferred color.

		Cellosolve/DMF			ABS Measurements from S
		Solvent Volume, uL		650	545
Process Control 1		100	Process Control Navy	1.4600	0.1024
Process Control 2		100	Process Control Navy	1.4600	0.1024
Process Control 3		100	Process Control Navy	1.4600	0.1024
			Process Control Results	Avg ABS	Avg Control Microsphers
				1.460	5000

Reference Blood Samples:

Example shown below:

Reference Blood	Reference Withdrawal	Cellosolve/DMF	Reference		ABS Measurements from S
Description	Rate,ml/min	Solvent Volume, uL	Color	650	545
Control	10.000	200	Persimmon	0.8760	1.3215
Ischemia	10.000	200	Tangerine	0.8760	0.3429
Treatment, Time 0 min	10.000	200	Orange	0.8760	0.0615
Treament, Time 30 min	10.000	200	Yellow	0.8760	0.0615
Treatment, Time 600 min	10.000	200	Lemon	0.8760	0.0615

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

Tissue Samples

Example shown below:

Tissue	Tissue	Cellosolve/DMF	Tissue	ABS Measurements from Spectrophoto	
Description	Weight,Grams	Solvent Volume, uL	Number	650	545
Liver	2.0000	200	T1	1.4600	0.3980
Kidney	3.0000	200	T2	1.3200	0.5550

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS

RBF Calculation Page

Regional Blood Flow calculations are performed automatically through links to data on the **Data Calculation** page.

Important Tip:

Delete unused rows by selecting the top unused row with the left button on the mouse. Click just off the spreadsheet on the far left row number column -'Black Number on a Grey Background' label.

Continue to hold the left button down and drag the mouse down over all other unused rows. Release the left mouse button.

Right click the mouse in the row number column and select the 'DELETE' option.

This method of deleting rows will allow the RBF Chart to automatically update only to the actual number of samples.

APPENDIX 'B' EXCEL SPREADSHEET INSTRUCTIONS