

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

(version 0606)

(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 loss in 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.

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 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, as it increases the accuracy of the blood flow measurement at the additional cost of only a few cents per sample.

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

Example: ***'TritonVII+ 6 Plus Control.xls'***

<i>'TritonVII+'</i>	The Dye-Trak VII+ family of microspheres
<i>'6 Plus Control'</i>	Six experimental colors plus a Process Control

Example: ***'TritonVII+ 6.xls'***

<i>'TritonVII+'</i>	The Dye-Trak VII+ family of microspheres
<i>'6'</i>	Six experimental colors (no control)

Example: ***'Triton4 Plus Control.xls'***

<i>'Triton'</i>	The original Dye-Trak family of microspheres
<i>'4 Plus Control'</i>	Four experimental colors plus a Process Control

Example: ***'Triton5.xls'***

<i>'Triton'</i>	The original Dye-Trak family of microspheres
<i>'5'</i>	Five experimental colors (no control)

File Archiving:

For a file to run the Excel macro modules, the file must have its original file name as shown in the above examples. Following the importation of absorbance data with the execution of the ***DyeTrak*** file transfer module, the Excel file should be saved with a new name that identifies the experiment so that the Regional Blood Flow data can be retrieved at any later time.

Example: **TritonVII+ 6 Plus Control.xls** might be saved as **Experiment 3-1.xls**

Critical: A backup copy of the original Excel file should always be saved in a separate archive directory.

Spreadsheet Cell Color convention:

The data input areas are divided into the following general groups according to cell background color. Verify that all data inputted 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.

Spreadsheet Page Overview:

* **Data Calculation** page

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 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. The average value of the three readings is used to determine 'percentage recovery' of the samples (**Data Calculation page, cell E43**).

The 'Reference Withdrawal' blood data is also entered on this 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 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 and the volume of solvent used to extract the dye from this sample and the sample identifying number that is on the sample tube. The absorbance values for each microsphere color associated with the sample are also entered on this sample line. 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.

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. 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 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. 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 'copy-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 Page**

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.

*** RBF Chart**

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

Spreadsheet Page Details:

File Data Page:

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

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 is 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* is 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* is a three character numeric, increasing sequentially from ".001", ".002", etc to the last sample 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**

Using the *DyeTrak* data transfer macro:

1. Select the appropriate spectrographic file structure for your spectrophotometer from the '*DyeTrak*' drop-down 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 other words, in the following sequence the macro will stop importing after the fifth file** as there is a gap in the sequence,:*

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 1nm steps, or read in 5nm steps, or read only the wavelengths listed on the **Data Calculation** page. If the spectrophotometer reads in 2nm steps the macro will fail when it 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

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

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

<u>File Ext</u>	<u>File Name</u>	<u>Sample Label</u>	<u>ABS Wavelengths from Spectrophotometer (nm) (Linked to Data Calculation Page: E27-J27)</u>					
			<u>650</u>	<u>545</u>	<u>525</u>	<u>495</u>	<u>440</u>	<u>390</u>
.001	C:\Data.001	T1	0.0010	0.0010	0.0010	-0.0010	0.0020	0.5510
.002	C:\Data.002	T2	0.0000	0.0010	0.0010	-0.0010	0.0020	0.9790
.003	C:\Data.003	T3	0.0000	0.0010	0.0010	0.0060	1.0260	0.2200
.004	C:\Data.004	T4	0.0060	0.0100	0.0210	1.4960	0.0120	0.0010
.005	C:\Data.005	T5	0.0040	0.3980	1.4450	0.5220	-0.0020	0.0370
.006	C:\Data.006	T6	0.0060	0.5550	0.3020	0.1170	0.0880	0.0100
.007	C:\Data.007	T7	1.1370	0.0770	0.0310	0.0070	-0.0020	0.0020

Data Calculation page:

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

I: Characterization of Microsphere Spectra Profiles

The Microsphere Spectra Profile table reflects the analysis of each individual microsphere color at each of the several color peaks for the experiment. Each experiment performed should have new Microsphere Spectral Profile measurements for all the colors used in the 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.

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/100uL 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.

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 1.5 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.

II: 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 Microspheres
				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
Treatment, Time 30 min	10.000	200	Yellow	0.8760	0.0615
Treatment, Time 600 min	10.000	200	Lemon	0.8760	0.0615

Tissue Samples

Example shown below:

Tissue	Tissue	Cellosolve/DMF	Tissue	ABS Measurements from Spectrophotometer	
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

RBF Calculation Page

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

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.