Multiplex assays with Alpha *SureFire Ultra* Multiplex technology using the EnVision multimode plate reader.

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Introduction

In this technical note, we will describe how to set up Alpha *SureFire® Ultra™* Multiplex kits on the EnVision™ multimode plate reader to measure Europium and Terbium signals and provide instructions for correcting the raw data obtained during each type of measurement.

Assay principle

The Alpha *SureFire Ultra* Multiplex assay kits enable the dual measurement of phosphoproteins from cells, combined with the measurement of the total amount of the same protein or of another phosphoprotein. This dual measurement is carried out in the same assay plate well from a single sample of cell lysate, and is achieved by the use of two types of Alpha Acceptor beads that emit at distinct wavelengths (AlphaPlex™ Terbium beads: 545 nm and AlphaLISA™ Europium beads: 615 nm).
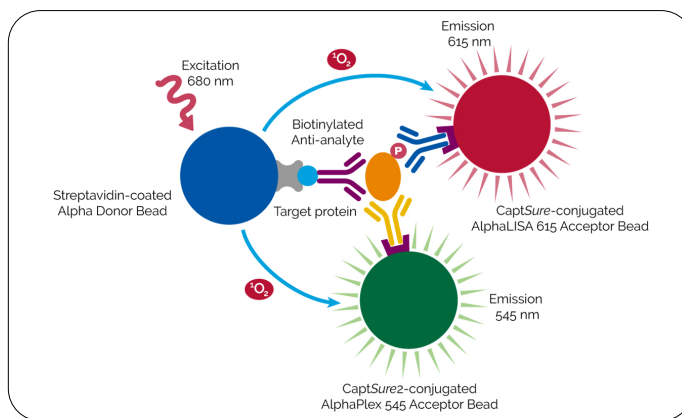

Figure 1: Schematic of Alpha *SureFire Ultra* Multiplex assay.

For research use only. Not for use in diagnostic procedures.

A schematic of the Alpha *SureFire Ultra* Multiplex assay format is shown in Figure 1. The AlphaLISA 615 Acceptor bead (Alpha 615 bead) is directly-conjugated with an antibody to the phosphorylated site on the target protein. The AlphaPlex 545 Acceptor bead (Alpha 545 bead) is coated with the CaptSure™ agent, which binds the CaptSure™ tagged anti-total target protein antibody. The Alpha Donor bead binds the biotinylated anti-total target protein antibody. The protocol is homogeneous, can be performed with or without a transfer step, and provides rapid and sensitive detection of phosphorylated and total protein in less than three hours.

Sample protocol

A typical protocol for using Alpha *SureFire Ultra* Multiplex kits is shown in Figure 2. The kits offer a wide range of flexibility in terms of assay set up. More versions of the protocol can be found in the product manuals which include variations for adherent and suspension cells, transfer vs. non-transfer protocol, and incubation times to improve assay sensitivity.
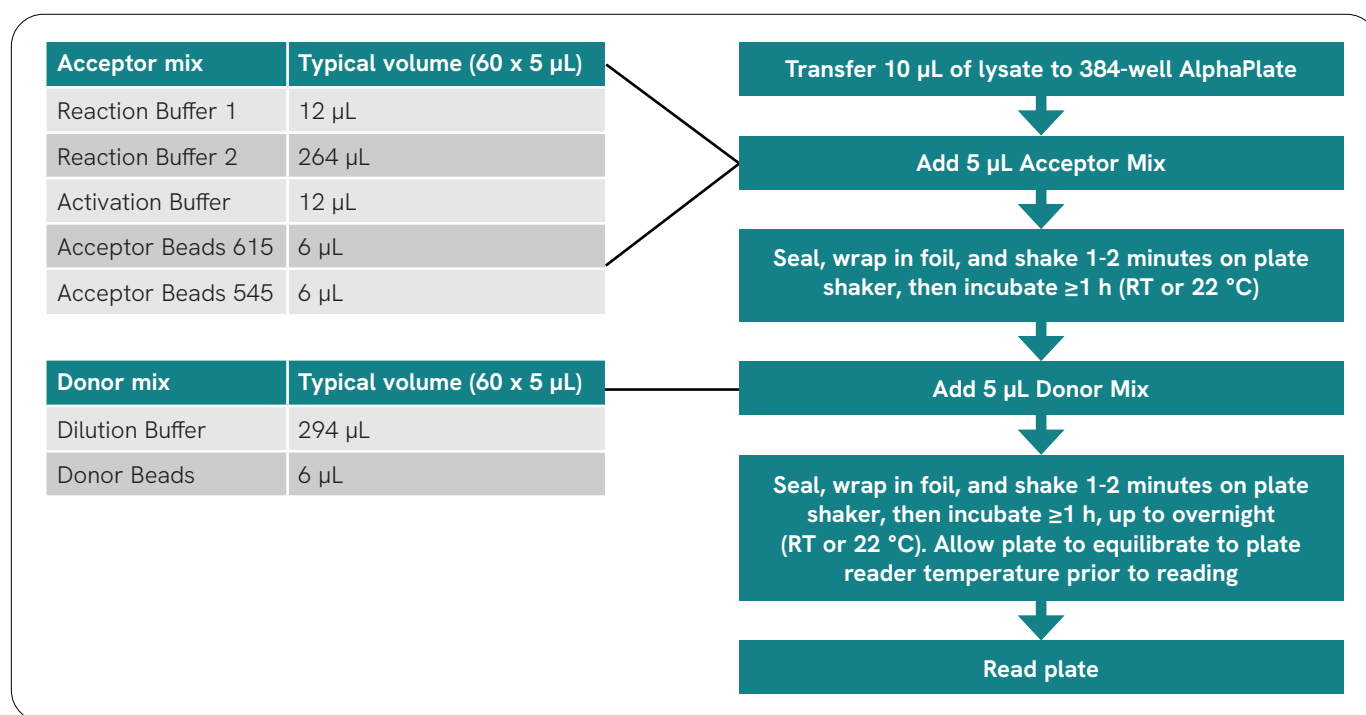
Acceptor mix	Typical volume (60 x 5 µL)
Reaction Buffer 1	12 µL
Reaction Buffer 2	264 µL
Activation Buffer	12 µL
Acceptor Beads 615	6 µL
Acceptor Beads 545	6 µL

Donor mix	Typical volume (60 x 5 µL)
Dilution Buffer	294 µL
Donor Beads	6 µL

Transfer 10 µL of lysate to 384-well AlphaPlate

↓

Add 5 µL Acceptor Mix

↓

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥1 h (RT or 22 °C)

↓

Add 5 µL Donor Mix

↓

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥1 h, up to overnight (RT or 22 °C). Allow plate to equilibrate to plate reader temperature prior to reading

↓

Read plate

Figure 2: Alpha *SureFire Ultra* Multiplex protocol for a 384-well plate.

EnVision hardware and software requirements

Not all models of the EnVision allow for reading of multiplexed Alpha technology, such as *SureFire Ultra* Multiplex kits. Table 1 explains which models are compatible with multiplexing Alpha technology. In order to measure multiplexed Alpha signals, a filterbased reader is required to distinguish the signal originating from Europium AlphaLISA beads from the signal originating from Terbium AlphaPlex beads. Software version 1.13 is required to read multiplexed Alpha signal. For instruments with previous versions of the software, an upgrade will be needed to enable reading. Please contact your Revvity service engineer for more information on software upgrades.

Table 1: Available EnVision Alpha options and multiplex capabilities.

EnVision Alpha option	EnVision Alpha option	Multiplex capable?	Reading mode
Alpha Standard (Filter-based)	1	Yes	Sequential only
	2	Yes	Simultaneous or Sequential
Alpha HTS (Aperturebased), Sometimes Referred to as "Turbo"	1 or 2	No	n/a

EnVision required optics

The EnVision requires AlphaPlex specific filters and mirrors to read *SureFire Ultra* Multiplex assays. A summary of the recommended optics is shown in Table 2.

Table 2: Optics required for reading multiplexed Alpha assays on the EnVision.

	Description	Cat. #	Bar-code	Recommended use
Mirrors	AlphaScreen Single	2101 - 4010	444	Sequential duplexing of Eu and Tb
	AlphaPlex Single Tb-Eu	2102 - 5910	605	For all sequential AlphaPlex applications
	AlphaPlex Dual Tb-Eu	2102 - 5900	653	Simultaneous duplexing of Tb and Eu
Filters	Europium	2100 - 5090	203	For all Eu applications
	Resorufine/Amplex Red	2100 - 5570	124	Suitable for Tb single plexing and Tb/Eu duplexing
	AlphaPlex Tb	2100 - 5930	701	For all Tb applications and multiplexing

Please note that if you have not previously used the optics you will need to add them as new inventory in the EnVision database.

Please see the Appendix for how to install new optics into the EnVision database.

Available plate reading modes

Currently available EnVision standard Alpha-enabled readers will either have one or two photomultiplier tubes (PMTs). EnVisions that have one PMT (i.e. the EnVision Xcite) are able to read AlphaPlex in sequential mode, meaning the plate is read once in one channel (e.g. Terbium or Europium) and then again in the second channel (e.g. Terbium or Europium). EnVision systems that have two PMTs are able to read in sequential mode or simultaneous mode. In simultaneous mode, both signals (Europium and Terbium) will be read simultaneously. This means each well is only read one time with a readout in both the Terbium and Europium channels. While reading the plate in sequential mode takes longer than simultaneous mode, the overall signal will be much higher if the plate is read in sequential mode.

Reading in sequential mode

When running multiplex assays with Europium and Terbium in sequential mode, the EnVision allows the user to select which chelate can be read first. The total light emission from the Terbium chelate is much weaker than the Europium chelate. Due to Terbium being the weaker light emitter, we recommend choosing a measurement mode that will give the highest possible signal in the Terbium channel, which requires the emission in the Terbium channel to be measured first. The alternate configuration can be used (reading Europium signal first), but this will result in an overall much lower Terbium signal which might not be ideal for assays. A summary of how to set up the EnVision for reading AlphaPlex in sequential mode (Terbium first) can be found in the Appendix.

Reading in simultaneous mode

When running multiplex assays with Terbium and Europium in simultaneous mode, it is important for the user to select the proper setup and orientations of the Terbium and Europium filters so that signal from each channel is directed to the correct PMT. The optimal optic setup allows for the Terbium signal to be directed to the first PMT and the Europium signal to be directed to the second PMT. This is due to the architecture of the "AlphaPlex Dual Tb/Eu" mirror module, which directs the Terbium signal to the first EnVision detector and the Europium signal to the second detector. Because this was how the mirror module was designed to work, it is important the filters are in the proper orientation. A summary of how to set up the EnVision for reading AlphaPlex in simultaneous mode can be found in the Appendix. A summary of data obtained in sequential and simultaneous reading modes is shown in Figure 3. While the level of signal is globally higher when performing sequential readings due to the filtering of part of the light by the mirror module used in simultaneous mode, the sensitivity and the quality of the assay is not impacted by this decrease. This is fully explained and exemplified in the Application Note "Simultaneous Detection of Total and Phosphorylated Protein in Cellular Kinase Assays using Alpha *SureFire Ultra* Multiplex Technology" (Revvity Document #012925).
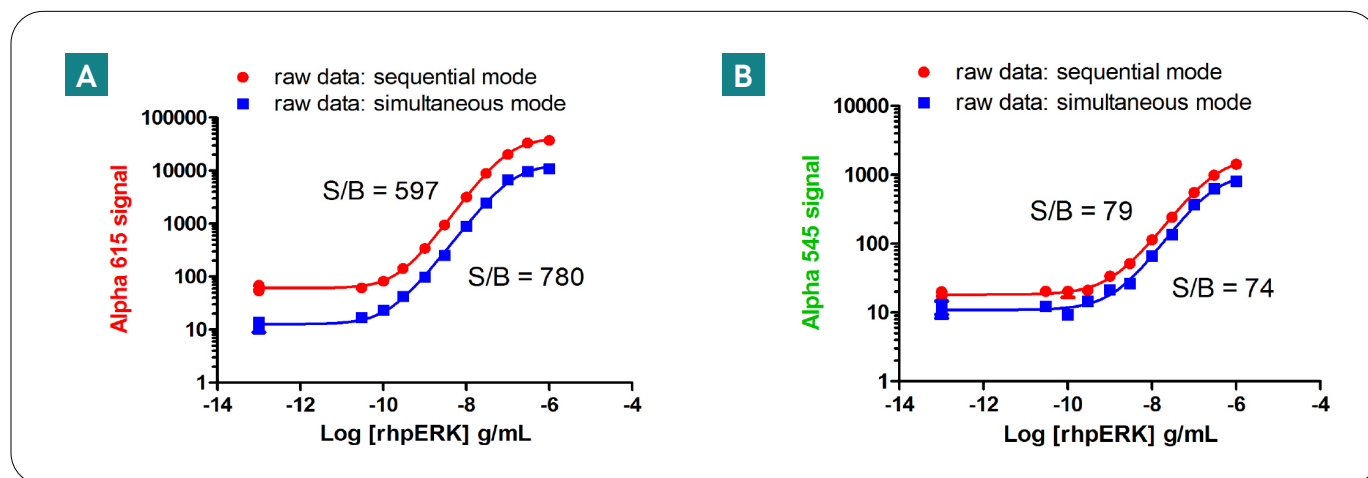
Figure 3: Standard curves using recombinant protein and the Alpha *SureFire Ultra* Multiplex kits. A) Europium signal (615 nm, for pERK detection and B) Terbium signal (545 nm, for total ERK detection) run in either sequential (red circles) or simultaneous (blue squares) mode

Spectral overlap correction

While the main peaks of emission spectra from Europium and Terbium are narrow and distinct, there is some minor overlap from one chelate to the other in the detection region of each chelate (545 nm for Terbium, 615 nm for Europium), as depicted in Figure 4. In order to remove the small contributions that each chelate contributes to the signal of the other chelate, a Spectral Overlap Correction factor (SOC) is needed.

We recommend to determine SOC per instrument (i.e. a SOC determined on a specific EnVision reader cannot be applied to a different unit) and per optics used. The amount of spectral overlap that occurs in the assay will vary slightly from instrument to instrument. We recommend running a control experiment, using the positive control lysates contained inside the *SureFire Ultra* Multiplex kit, to calculate the amount of spectral overlap and then applying this factor

to the raw data obtained from the EnVision reader. A sample plate layout along with how to prepare additional control detection mixes is shown in Figure 5.
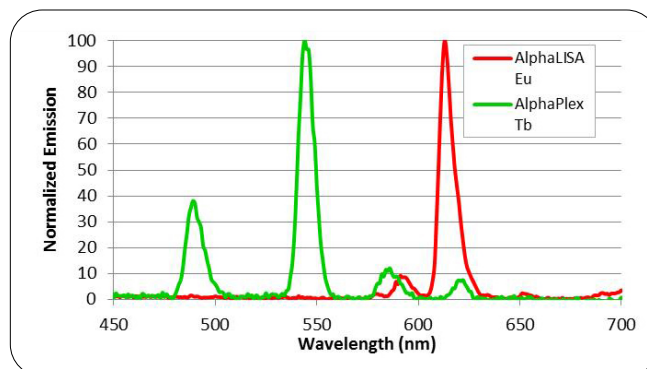

Figure 4: Representative sample spectra of AlphaLISA Europium and Terbium. Please note that the values plotted are "Normalized Emission" only as the true measured signal of the Terbium signal is weaker than that of the Europium signal.

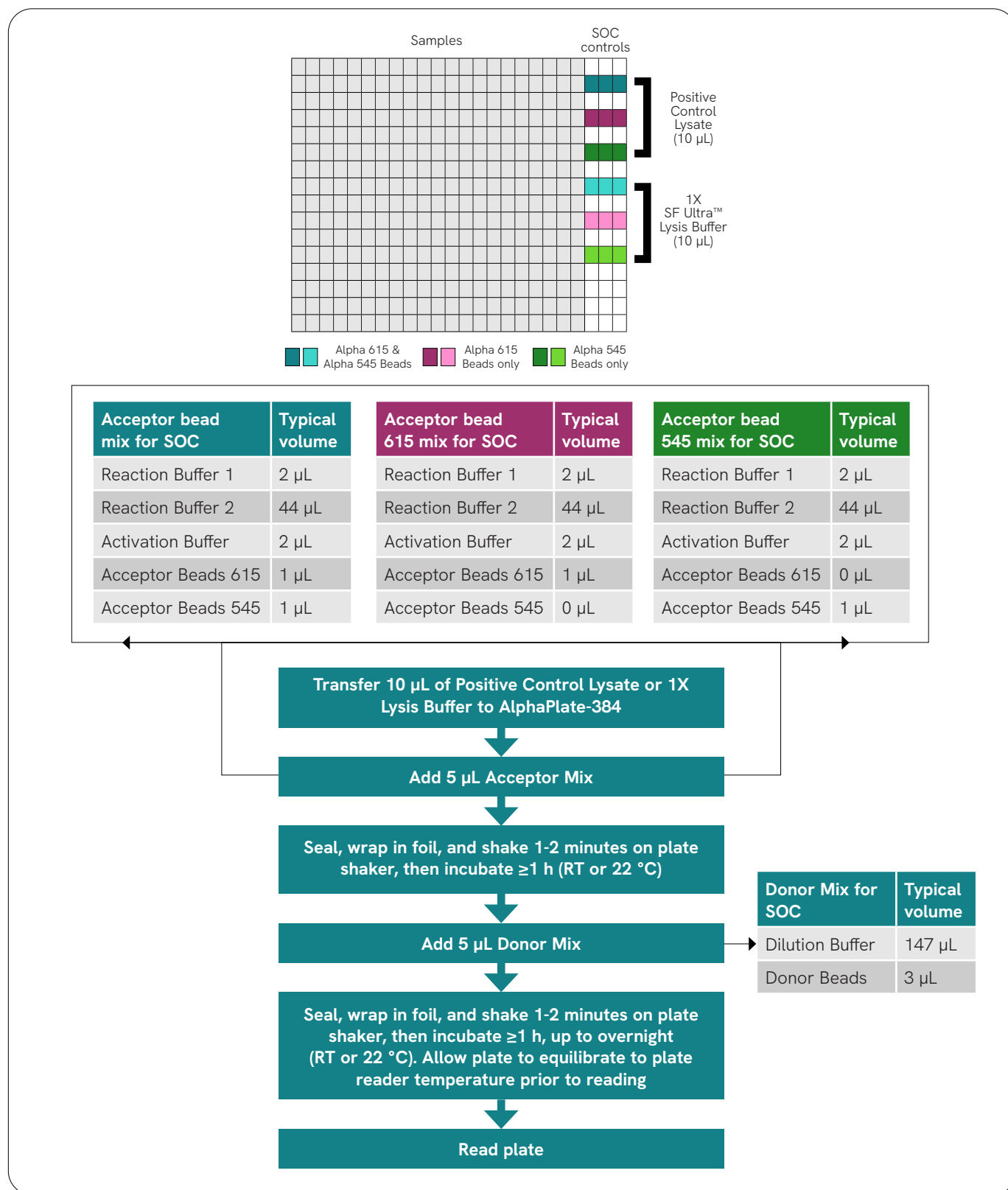## Preparing controls to measure spectral overlap correction

Acceptor bead mix for SOC	Typical volume
Reaction Buffer 1	2 µL
Reaction Buffer 2	44 µL
Activation Buffer	2 µL
Acceptor Beads 615	1 µL
Acceptor Beads 545	1 µL

Acceptor bead 615 mix for SOC	Typical volume
Reaction Buffer 1	2 µL
Reaction Buffer 2	44 µL
Activation Buffer	2 µL
Acceptor Beads 615	1 µL
Acceptor Beads 545	0 µL

Acceptor bead 545 mix for SOC	Typical volume
Reaction Buffer 1	2 µL
Reaction Buffer 2	44 µL
Activation Buffer	2 µL
Acceptor Beads 615	0 µL
Acceptor Beads 545	1 µL

Transfer 10 µL of Positive Control Lysate or 1X Lysis Buffer to AlphaPlate-384

↓

Add 5 µL Acceptor Mix

↓

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥1 h (RT or 22 °C)

↓

Add 5 µL Donor Mix

Donor Mix for SOC	Typical volume
Dilution Buffer	147 µL
Donor Beads	3 µL

↓

Seal, wrap in foil, and shake 1-2 minutes on plate shaker, then incubate ≥1 h, up to overnight (RT or 22 °C). Allow plate to equilibrate to plate reader temperature prior to reading

↓

Read plate

Figure 5: Plate Layout and Acceptor Bead Mixes to Calculate SOC. Two additional bead mixes are prepared in addition to the mix used to test samples, one containing only Alpha 615 nm (Europium) beads and one containing only Alpha 545 nm (Terbium) beads. Note that the volumes recommended for Acceptor and Donor bead mixes are just enough to run the control wells. If samples are run alongside the SOC controls, additional mixes of Alpha 545 and Alpha 615 Acceptor and Donor beads will be needed, according to the flowchart in Figure 2.

Calculating the spectral overlap correction factor

After reading the samples and controls for Alpha *SureFire Ultra* Multiplex, SOC can be calculated. Sample raw data, generated using the protocol in Figure 5, are shown in

Table 3. Data are obtained for the positive control lysates as well as the 1X lysis buffer as a background control.

Table 3: Sample Raw Data obtained in the Terbium and Europium channels for SOC controls.

Raw data	Terbium channel			Europium channel		
	Alpha 615 and Alpha 545 beads			Alpha 615 and Alpha 545 beads		
Positive Control Lysate	862	748	735	18499	15770	16758
1X SF *Ultra* Lysis Buffer	14	13	17	17	22	16
	Alpha 615 beads Only			Alpha 615 beads Only		
Positive Control Lysate	225	220	206	17001	15946	15807
1X SF *Ultra* Lysis Buffer	6	9	6	22	25	22
	Alpha 545 beads Only			Alpha 545 beads Only		
Positive Control Lysate	660	683	706	19	12	16
1X SF *Ultra* Lysis Buffer	20	21	16	4	4	4

The first step is to average the values obtained for the positive control lysates and the 1X lysis buffer for both the Europium and Terbium channels containing each bead type. We recommend subtracting the average background counts from the average positive control lysate counts for each condition. After averaging the positive control lysate signal and subtracting out the background, the data now look as shown in Table 4:

Table 4: SOC data from Table 3, with background data from lysis buffer subtracted from the positive control lysate data, and triplicate values averaged.

Average values and background subtract	Terbium channel	Europium channel
Positive Control Lysate	Alpha 615 beads only	Alpha 615 beads only
	210	16223
Positive Control Lysate	Alpha 545 beads only	Alpha 545 beads only
	664	12

Next, the signal from Alpha 615 nm beads in the Terbium channel is divided by the signal from the Alpha 615 nm beads in the Europium channel (Table 5).

This gives a value of 0.013, or 1.3%. This is the SOC factor to correct for the signal from Europium Alpha 615 nm beads contributing to signal in the Terbium channel.

Table 5: Using the 615 nm bead data from Table 4, the signal in the Terbium channel is divided by the signal from the same samples read in the Europium channel.

Average values and background subtract	Terbium channel	Europium channel	SOC (Ratio) Eu/Tb	SOC (%)
Positive Control Lysate	Alpha 615 beads only	Alpha 615 beads only		
	210	16223	0.013	1.3

Next, the signal from Alpha 545 nm beads in the Europium channel is divided by the signal from the Alpha 545 nm beads in the Terbium channel (Table 6).

This gives a value of 0.019, or 1.9%. This is the SOC factor to correct for the signal from Terbium Alpha 545 beads contributing to signal in the Europium channel.

Table 6: Using the 545 nm bead data from Table 4, the signal in the Europium channel is divided by the signal from the same samples read in the Terbium channel.

Average values and background subtract	Terbium channel	Europium channel	SOC (Ratio) Eu/Tb	SOC (%)
	Alpha 545 nm beads only	Alpha 545 nm beads only		
Positive Control Lysate	664	12	0.019	1.9

In Table 7, an example of typical SOC factors for each channel in each reading mode is shown. Regardless of the reading mode, the SOC factor will be slightly higher for Terbium into the Europium channel compared to Europium into the Terbium channel. Because the relative counts for Europium are significantly higher than that of Terbium in AlphaPlex assays, the amount of adjustment that occurs when applying the SOC to sample data will be greater to the Terbium samples than to the Europium samples.

Table 7: Sample SOC values obtained in each reading mode.

Reading mode	Eu in Tb	Tb in Eu
Sequential	1.1%	3.1%
Simultaneous	2.0%	3.6%

Applying the spectral overlap correction factor - Sample calculation

Once the Spectral Overlap Correction factor values have been established, they can be applied to the data obtained for the positive control lysates run in duplex mode (with both Alpha 615 and Alpha 545 beads), as well as the assay samples within the plate. A sample calculation (from the raw data shown in Tables 3-6) is shown in Table 8.

Table 8: Sample calculation of how to remove the spectral overlap from the Europium signal into the Terbium channel (left column) and from the Terbium signal into the Europium channel (right column).

Application of SOC Factor	Terbium channel	Europium channel
	Alpha 615 and Alpha 545 beads	Alpha 615 and Alpha 545 beads
Positive Control Lysate (Uncorrected)	782	17006
Positive Control Lysate - SOC Correction	782 – (17006*0.013)	17006 – (782*0.019)
Positive Control Lysate - SOC Correction	782 – 221	17006 – 15
Positive Control Lysate - SOC Corrected Signal	561	16991

Effect of reading mode on assay performance (with and without SOC)

Reading mode has very little effect on the overall assay performance of the Alpha *SureFire Ultra* Multiplex kits. The application of the SOC to the data collected will have minor effects on the data obtained in the Europium channel, but will have a larger effect on the data collected in the Terbium channel. This is due to the fact that the signal of the Europium chelate is higher than that of the Terbium chelate, therefore, the SOC changes the data in the Terbium channel more significantly than that in the Europium channel. As shown in Table 9, the EC_{50} and LDL of the pERK assay (Europium channel) are very similar regardless of whether the SOC factor is applied to the raw data. The EC_{50} and LDL of the tERK assay (Terbium channel) change more significantly when the SOC factor is applied to the raw data. This will be the case for whichever protein is detected in the Terbium channel.

Table 9: Alpha *SureFire Ultra* Multiplex assays analyzed without (top panel) and with (bottom panel) the SOC factor applied to the raw data. LDL refers to the lower detection limit of the assay and is calculated by interpolating the value of the background counts plus three times the standard deviation off of the standard curve.

pERK	EC_{50} (ng/mL)	LDL (pg/mL)	tERK	EC_{50} (ng/mL)	LDL (pg/mL)
Sequential	103	135	Sequential	300	530
Simultaneous	131	100	Simultaneous	227	1180
pERK SOC Corrected	EC_{50} (ng/mL)	LDL (pg/mL)	tERK SOC Corrected	EC_{50} (ng/mL)	LDL (pg/mL)
Sequential	103	135	Sequential	624	690
Simultaneous	132	102	Simultaneous	442	1893

Effect of SOC on compound IC_{50} assays

The influence of the SOC on the data obtained in the Europium channel is very minor. As such, compound IC_{50} measurements are very similar whether the SOC is applied to the data in the Europium channel or not. As shown in Figure 6, IC_{50} values for the two inhibitors shown are nearly identical in the pERK assay when using the Alpha 615 beads.
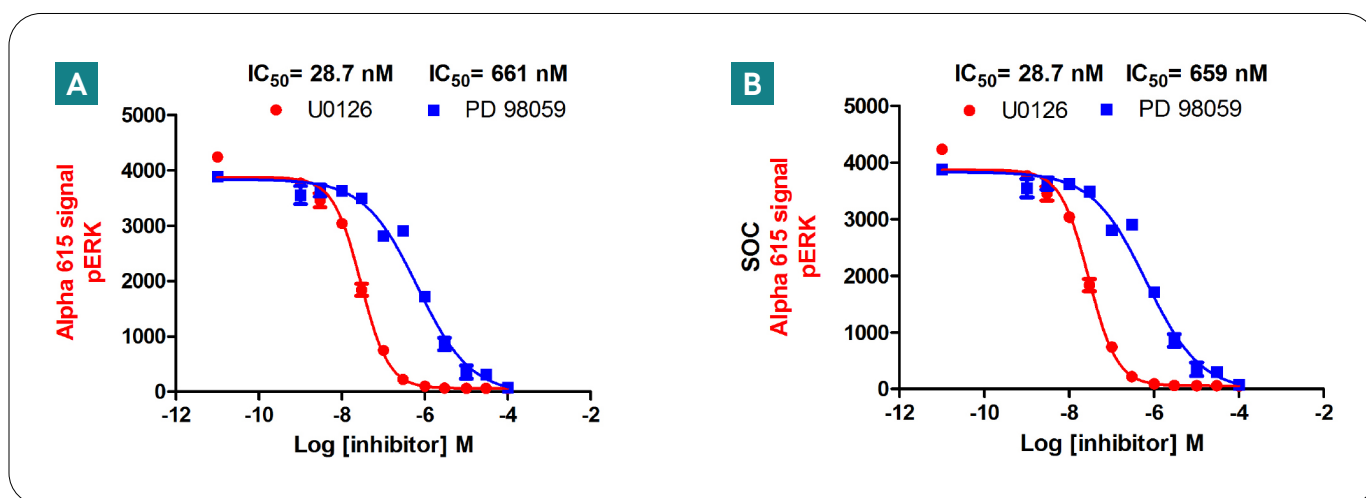
Figure 6: IC_{50} values calculated (A) without or (B) with the spectral overlap correction (SOC) factor.

Conversely, the application of the SOC to data obtained in the Terbium channel is much more significant and affects the interpretation of the data more critically. In the example below, total ERK signal is measured in the presence of increasing inhibitor concentrations. In this case, one would expect the signal in the Terbium channel to be constant regardless of inhibitor concentration. In Figure 7A, without SOC correction, there is a decrease in signal that correlates to the increase in inhibitor concentration.

This might imply that the compound is having an effect on total ERK levels within the samples tested. However, in Figure 7B, once the SOC correction is applied the curve flattens out. This indicates that the change in signal observed in Figure 7A was simply due to higher corresponding Europium counts in the samples with higher Europium signal (higher pERK levels) which was being observed in the Terbium channel due to spectral overlap.
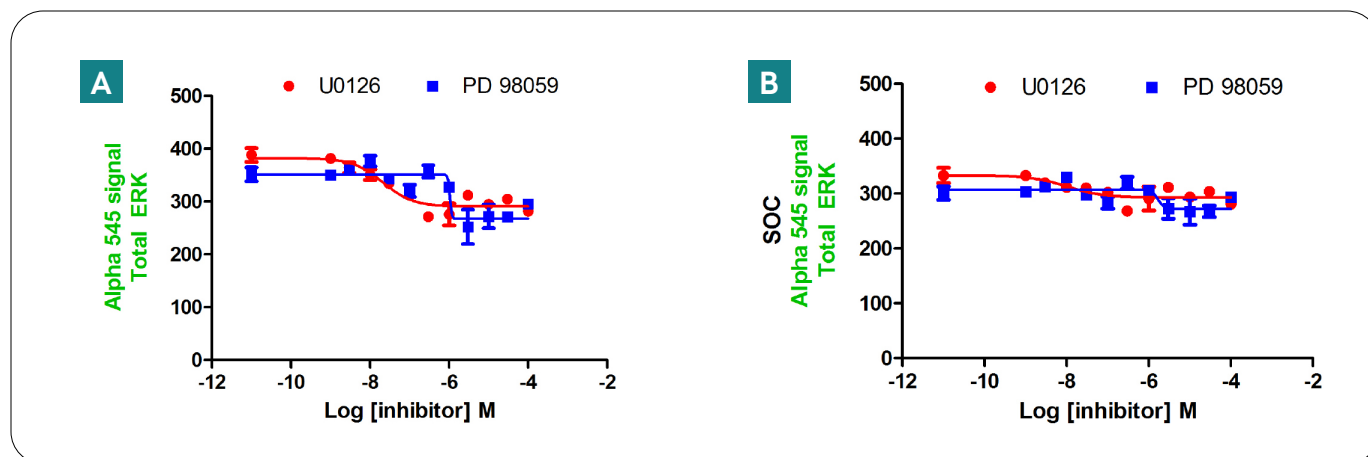
Figure 7: Total ERK detection in the Terbium channel (A) without or (B) with the spectral overlap correction (SOC) factor.

Effect of SOC and data normalization on Z' factor

Z' analysis is an important measure of assay performance and suitability for high throughput screening campaigns. A high Z' value (typically >0.6) is usually required in order to be sure signal responses generated by unknown compounds are statistically relevant. In cell-based assays, variability in cell numbers from well- to-well usually reduce assay Z'. In Alpha *SureFire Ultra* Multiplex assays, differences in cell numbers can be observed by differences in the total protein (e.g. ERK or AKT) values obtained in the Terbium channel. Normalization of phosphorylated protein (e.g. pERK and pAKT) values in the Europium channel to values obtained in the Terbium channel for total ERK and total AKT should help reduce assay variability originating from differences in cell number. In Table 10, sample Z' values improve after normalizing the signal from the Europium channel to the Terbium channel.

Table 10: Z' values obtained using the data in the Alpha 615 nm (Europium) channel alone or normalized to data from the Alpha 545 (Terbium) channel. Data obtained in sequential and simultaneous reading modes.

Z' values	Sequential reading	Simultaneous reading
pAKT only Alpha 615 (Europium)	0.81	0.64
pAKT/tAKT Alpha 615 (Europium)/ Alpha 545 (Terbium)	0.85	0.72

Applying the SOC factor to the data obtained in the Europium and Terbium channels has little effect on the Z' values obtained. Table 11 shows the same samples from

Table 10 analyzed after calculating the SOC and correcting the Europium and Terbium data for spectral overlap. Because the amount of spectral overlap does not affect the variability of the assay counts among duplicate samples (in most cases), the Z' values do not significantly change after applying the SOC to the data obtained in each channel.

Table 11: Z' values obtained using data in the Alpha 615 (Europium) channel alone (SOC corrected) or normalized to data from the Alpha 545 (Terbium) channel (SOC corrected). Data obtained in both simultaneous and sequential reading modes.

Z' values after SOC	Sequential reading	Simultaneous reading
pAKT only SOC Alpha 615 (Europium)	0.81	0.65
pAKT/tAKT SOC Alpha 615 (Europium)/ Alpha 545 (Terbium)	0.84	0.72

SOC vs. crosstalk correction

While SOC is used to correct for the overlap that occurs between spectra of the chelates used for multiplexed Alpha technology, "crosstalk correction" is often the term used to correct for the overlap in signal that occurs between adjacent wells of microplate-based assays. We have found that using AlphaPlates (Revvity # 6005350), which are slightly grey in color, significantly decreases the amount of well-to-well crosstalk (compared to white microplates) and reduces the needs to correct for well-to-well crosstalk in Alpha assays.

Summary and recommendations

When using Alpha *SureFire Ultra* Multiplex, it is important to calculate the spectral overlap correction factor (SOC) for each assay and instrument being used. SOC values will vary slightly by instrument and by assay. We recommend running SOC controls and calculating SOC to be sure the instrument settings you use are correct.

If the goal of using Alpha *SureFire Ultra* Multiplex is to screen compounds that have only an effect only on the Europium signal, the rank order of compound potency and relative compound IC_{50}s will remain the same with or without the application of the SOC.

If the goal of using Alpha *SureFire Ultra* Multiplex assays is to perform pharmacology, measure IC_{50}s in both Europium and Terbium channels, determine toxicity, benefit from absolute assay sensitivity, or to normalize signal from the Europium channel to the Terbium channel (to reduce assay variation), we recommend running SOC controls, calculating the SOC factor, and applying the SOC factor to the data obtained in both the Europium and Terbium channels before interpreting the experimental results.

Appendix - How to install hardware and set up protocols to read multiplexed alpha assays on the EnVision plate reader

Step one: Installation of the hardware

The first step is to install the mirror and filters. In order to do this, follow the instructions below.

1. Open the lid of the instrument. Once open, wait for the emission and excitation filter holders to come out.

2. Install the Emission filters 203 and 124 in the holder emission filter slider next to each other in that order (see figure below).

3. Install 444 (for sequential reading) and 653 (for simultaneous reading) mirrors on the mirror holder carousel. The exact position on the carousel is not critical.

4. Close the lid of the instrument.

5. On the reader control dialog box, a prompt will ask "Add filter [Name] to inventory?". Click "yes" to confirm. Repeat as needed for every part added. [This step is optional for demo filters only].

6. In the reader control on the computer, double-click on "Inventory". The following window will appear, confirming that the elements are installed.
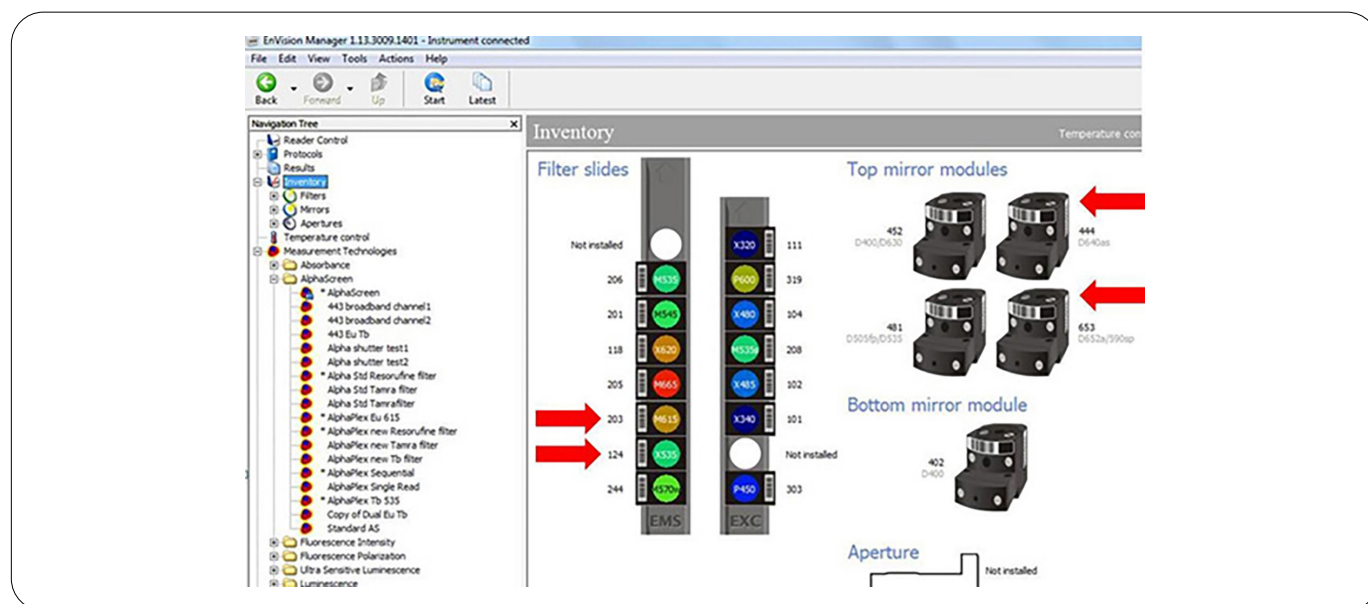
7. In the reader control open the "Inventory" section.

8. To create the mirror block 653 in Inventory, apply the following:

    a. Double click on the "Mirror" section.

    b. The following window will then appear:
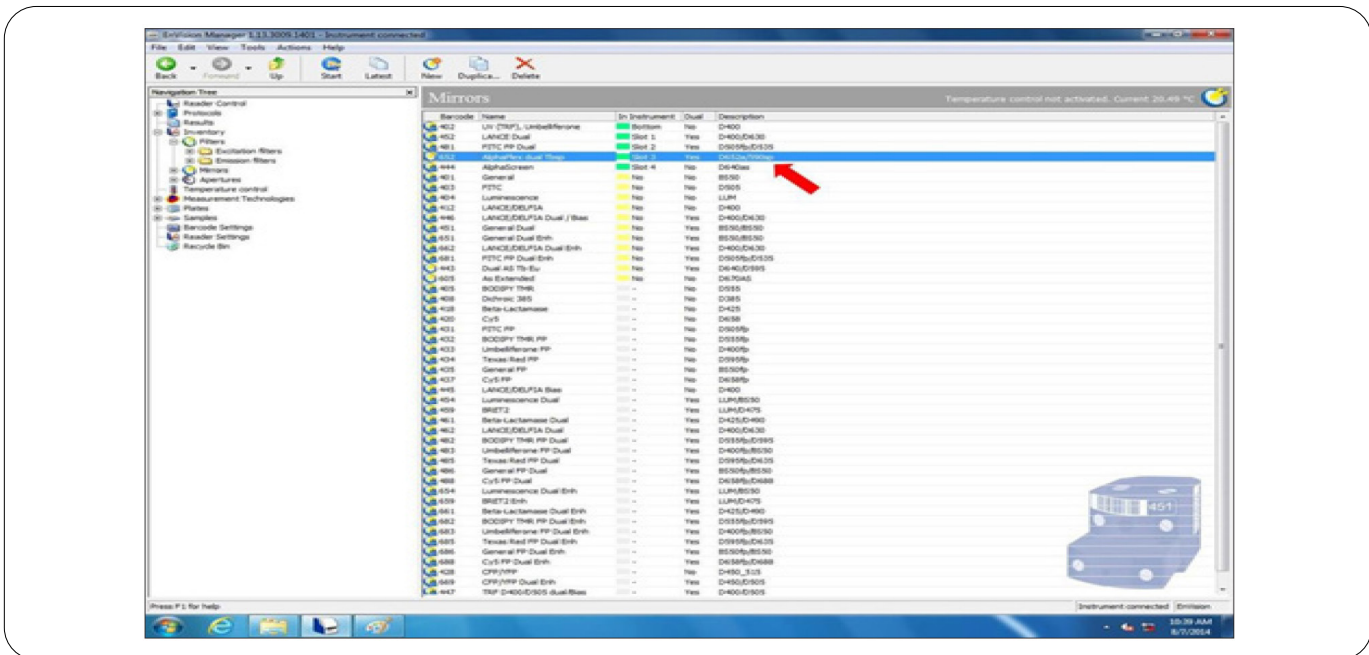
    c. Double click on the mirror that has just been added.

    d. A window similar to this will appear:
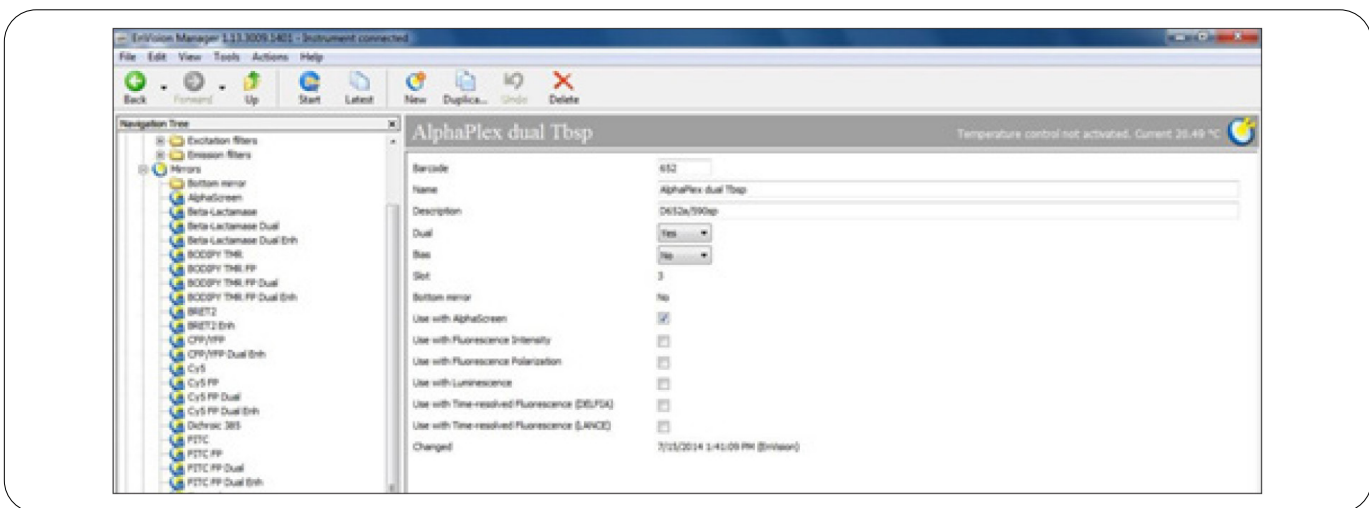
    e. Insert the following information:

        i. Name: AlphaPlex mirror 1.

        ii. Description: Terbium/Europium Dual Mirror.

        iii. In the dual slot put yes.

        iv. In the bias slot put yes.

        v. Check the "Use with AlphaScreen" box.

9. In the reader control, open the "Inventory" section.

10. Select the "Filter" section.

11. Double click on "Filter 203".

12. Check the "Use with AlphaScreen" box

13. Repeat for filter 124.

14. The instrument is now ready to be programmed.

Step two: Programming the reader for sequential reads

There are two options for reading a duplex assay: using sequential reads (reading the Terbium then the Europium channels) or doing both in a single read.

This first section describes how to program a sequential read.

The first step is to create the measurement methods (one for each wavelength). The following procedure should be followed.

1. On the reader control, go to Measurement Technologies.

2. Open the AlphaScreen folder.

3. Right click on "AlphaScreen" (the version with a small padlock)

4. Select "Duplicate".
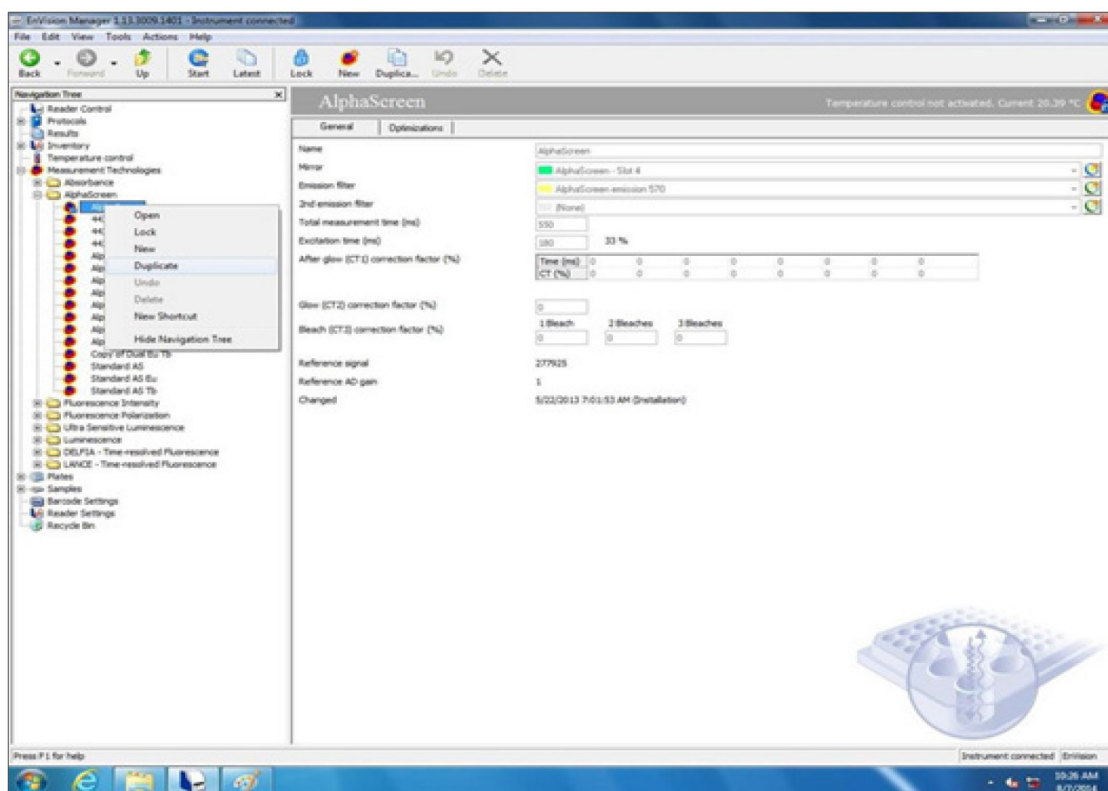
5. A copy of AlphaScreen will be created in the AlphaScreen measurement folder.

6. The following window will open:
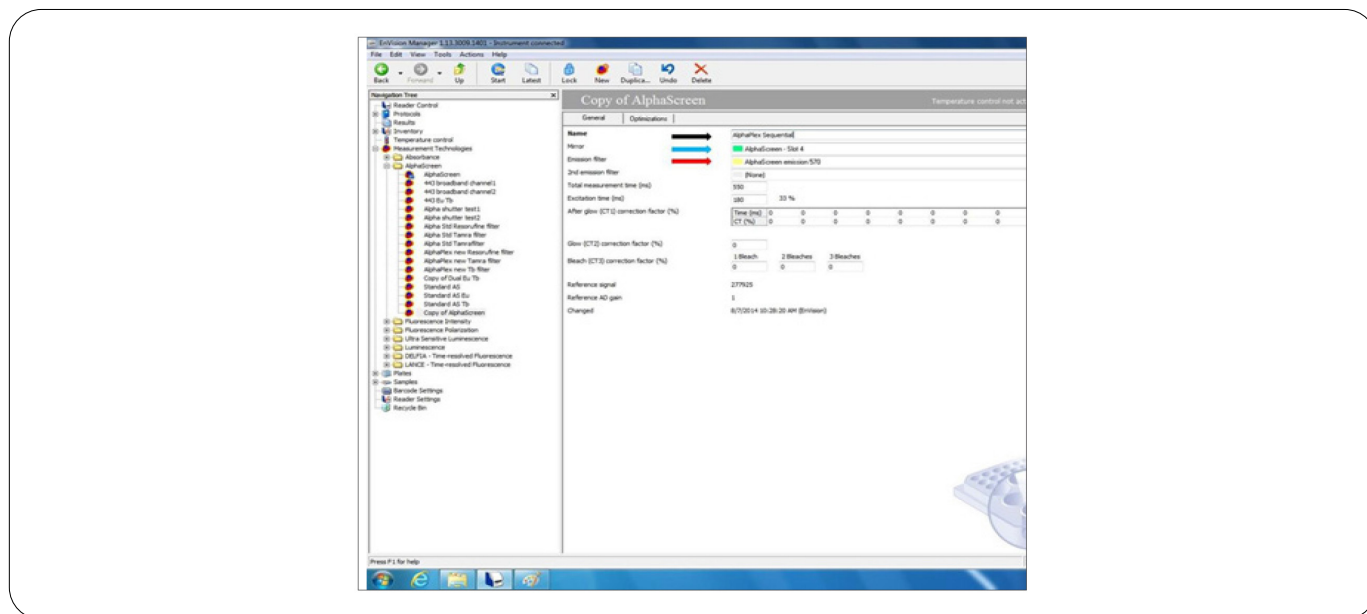
7. In the name box, enter the desired name. Recommended: "AlphaPlex 545 nm". ➡

8. In the mirror list, select "AlphaScreen". This will select the regular Alpha mirror, which is optimized for sequential reads. ➡

9. In the emission filter list, select filter "Amplex Red/Resorsufine 535". ➡

10. In version 1.13 of the software, a second emission filter box will appear if the reader is equipped with two PMTs For sequential reads leave the box empty.

11. Click "Back" button at the top of the window. The method will automatically save.

12. Repeat steps 3 to 10 using "Alpha 615 sequential" as the Name, and select "Europium 615" as the emission filter.

The second part is actually programming the method. The following steps should be taken:

1. In the Reader Control box, open the Protocols folder.

2. Right click on "AlphaScreen 384 Optiplate" (with the small padlock).

3. Select "Copy".
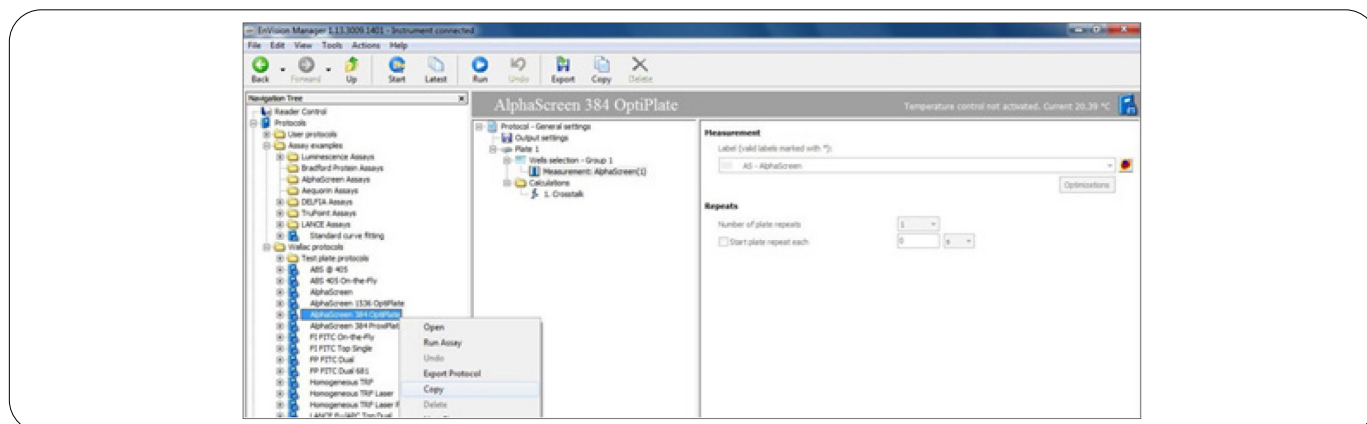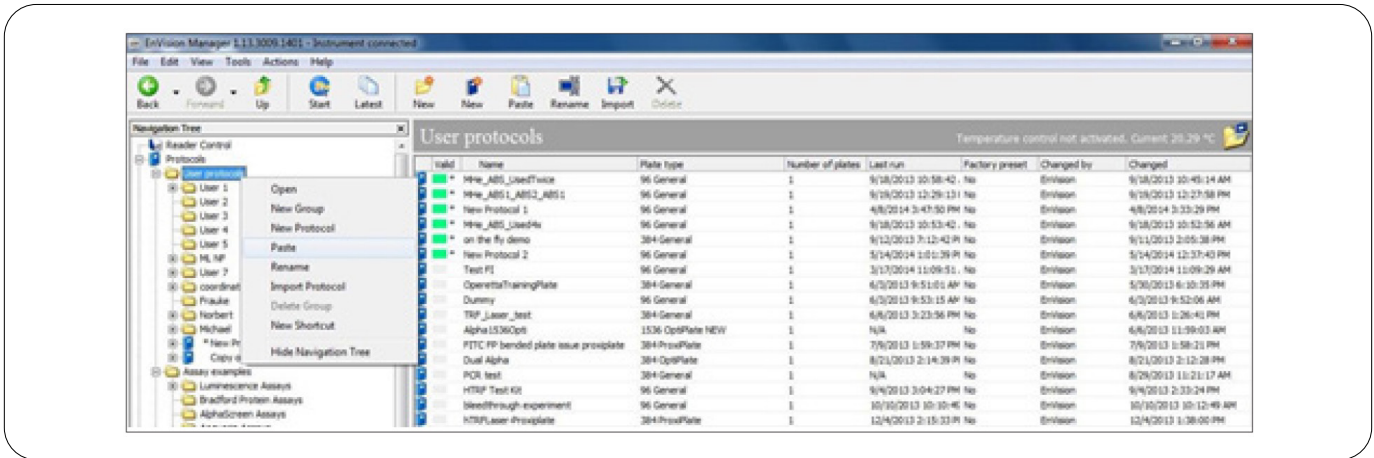
4. In the reader control right click "User protocols".

5. Select "Paste"

6. Double click on the new "Copy of AlphaScreen 384 OptiPlate" protocol

7. Click on "General Settings"

8. In the Name section give it the name you want.

9. In the Notes section note "AlphaPlex sequential reads".

10. In the Plate section, select the type of plate you want to use.
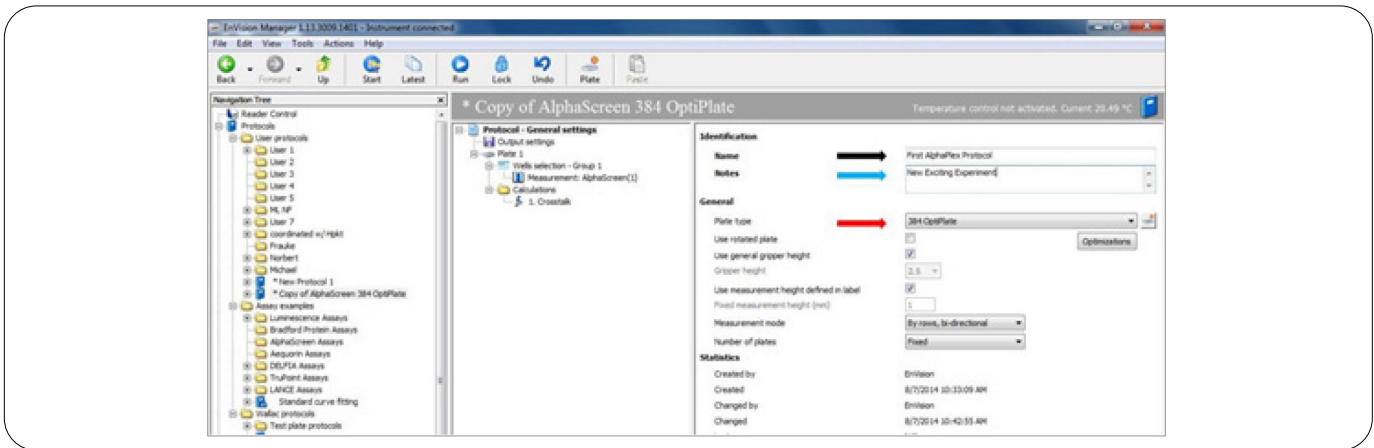
11. Click on "Measurement".
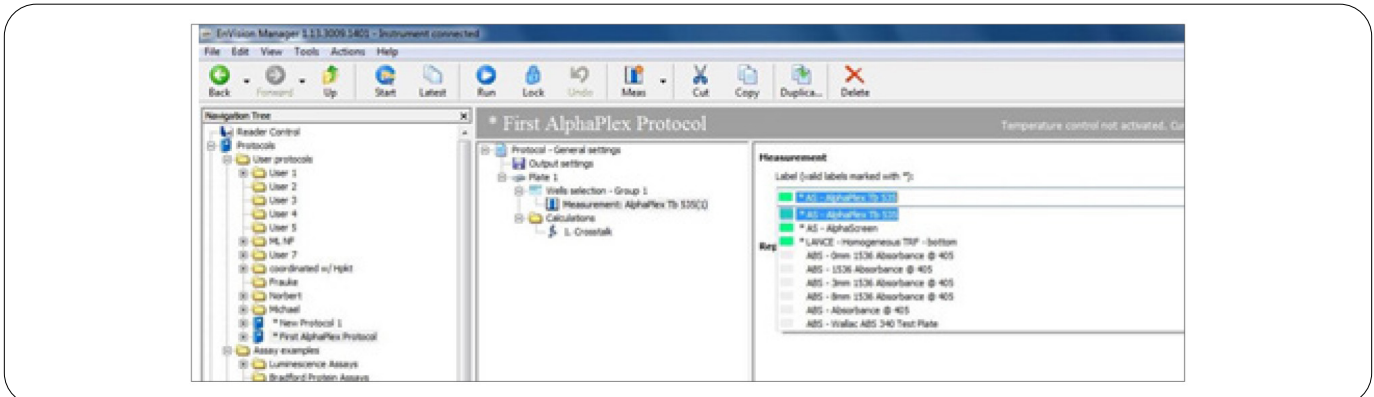
12. In the measurement label list, select "Alpha 545 sequential".

13. Click on "Measurement" again. Then click on "Duplicate". A second measurement will appear.

14. Click on the second measurement.
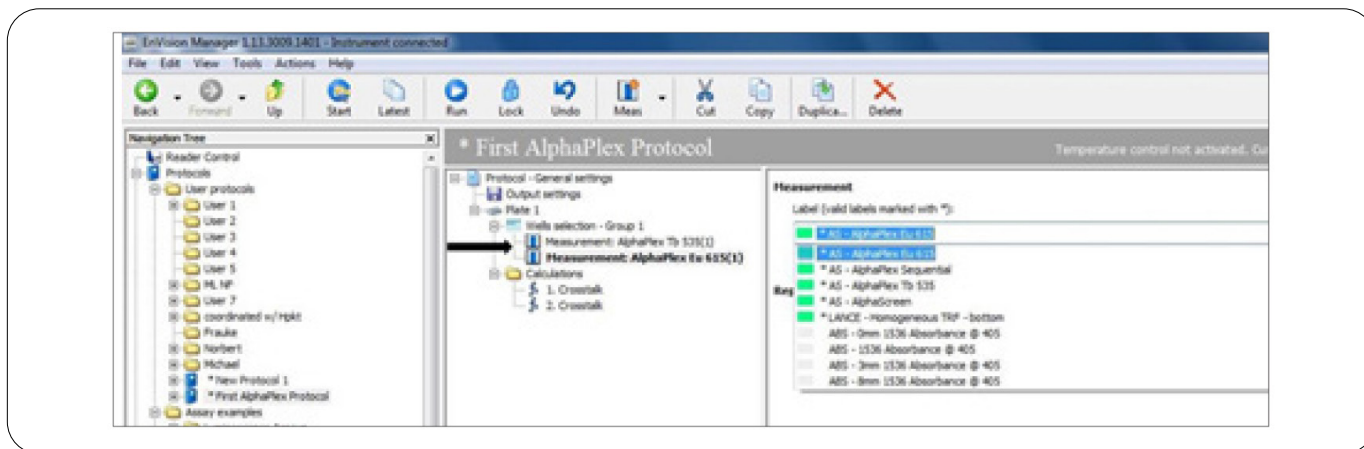
15. In the measurement label list select "Alpha 615 sequential".

16. Click the "Back" button at the top of the window. The protocol will automatically save.

17. If all necessary filters are present, the new protocol name will be followed by an asterisk (*).

Step three: Programming the reader for a simultaneous read

The second option is to use a simultaneous read of both channels. To create this, the technique is as follow:

The first part is to create the measurement methods (one for each wavelength). The following procedure should be followed.

1. On the reader control, go to Measurement Technologies.

2. Open the AlphaScreen folder.

3. Right click on "AlphaScreen" (the version with a small padlock)

4. Select "Duplicate".
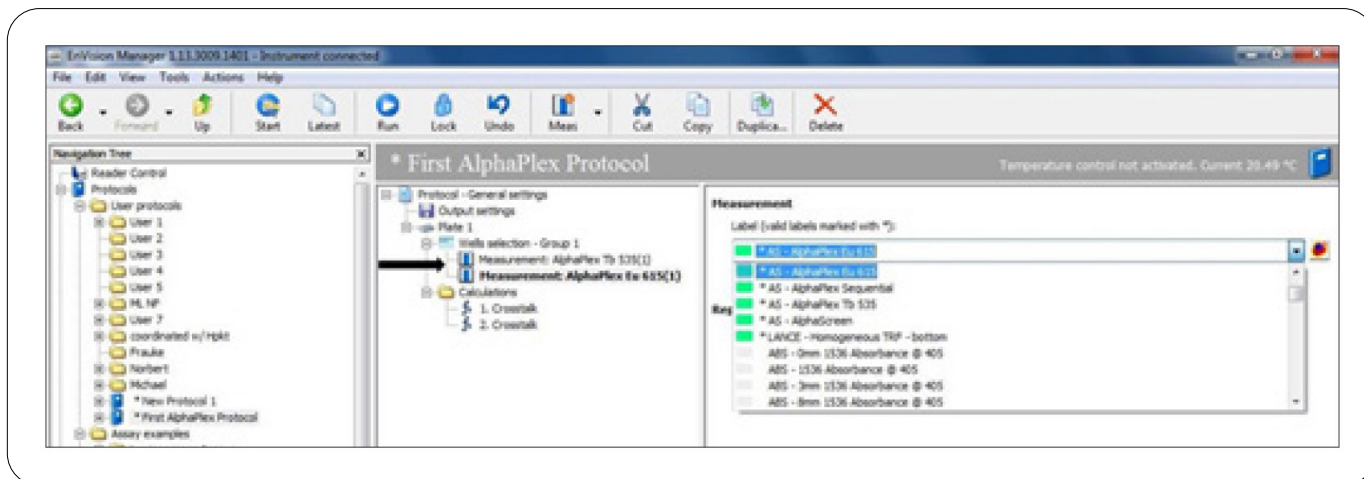
5. A copy of AlphaScreen will be created in the AlphaScreen measurement folder. Double click on it.

6. The following window will open:
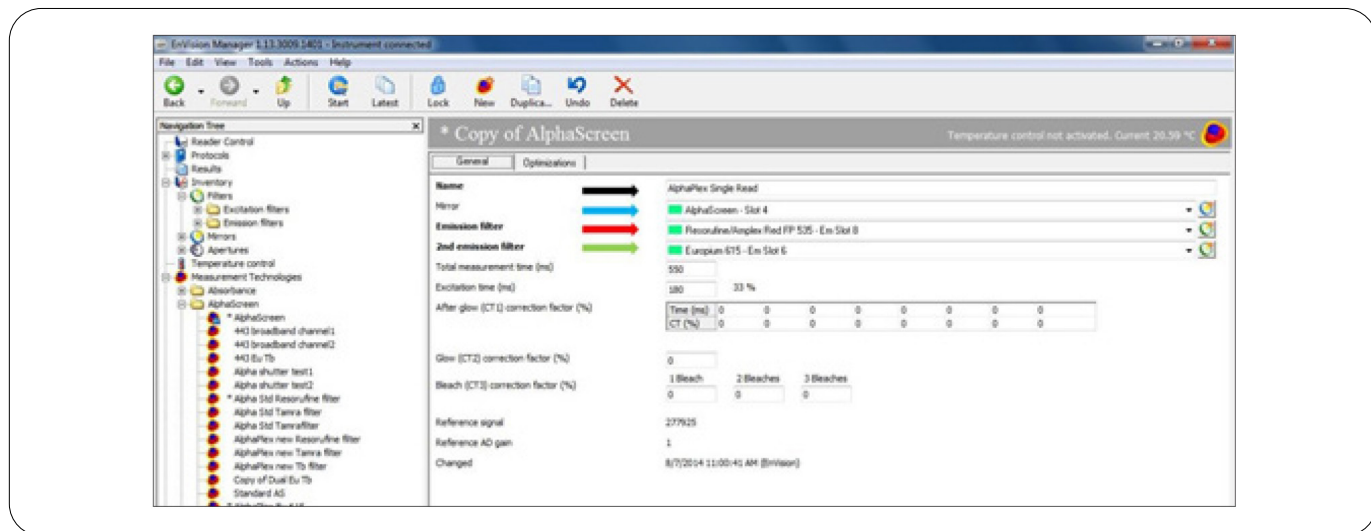
7. In the name box, enter the desired name. Recommended would be: "AlphaPlex Simultaneous Read".

8. In the mirror list, select "AlphaPlex mirror 1".

9. In the emission filter list, select "545 AlphaPlex filter".

10. In the second emission filter list, select "615 AlphaPlex filter".

11. Click the "Back" button at the top of the window. The method will automatically save.

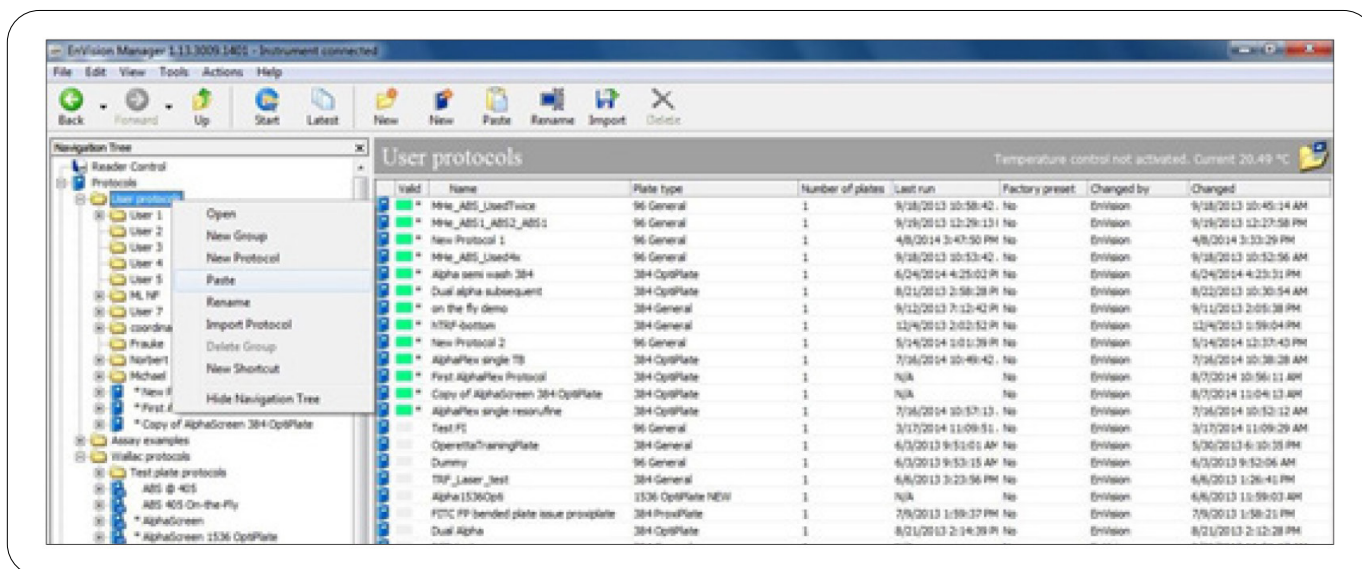The second step is to create the protocol.

In the reader control box, open the protocols folder:

12. In the reader control box, open the Protocols folder.

13. Right click on "AlphaScreen 384 Optiplate" (with the small padlock).

14. Select "Copy.
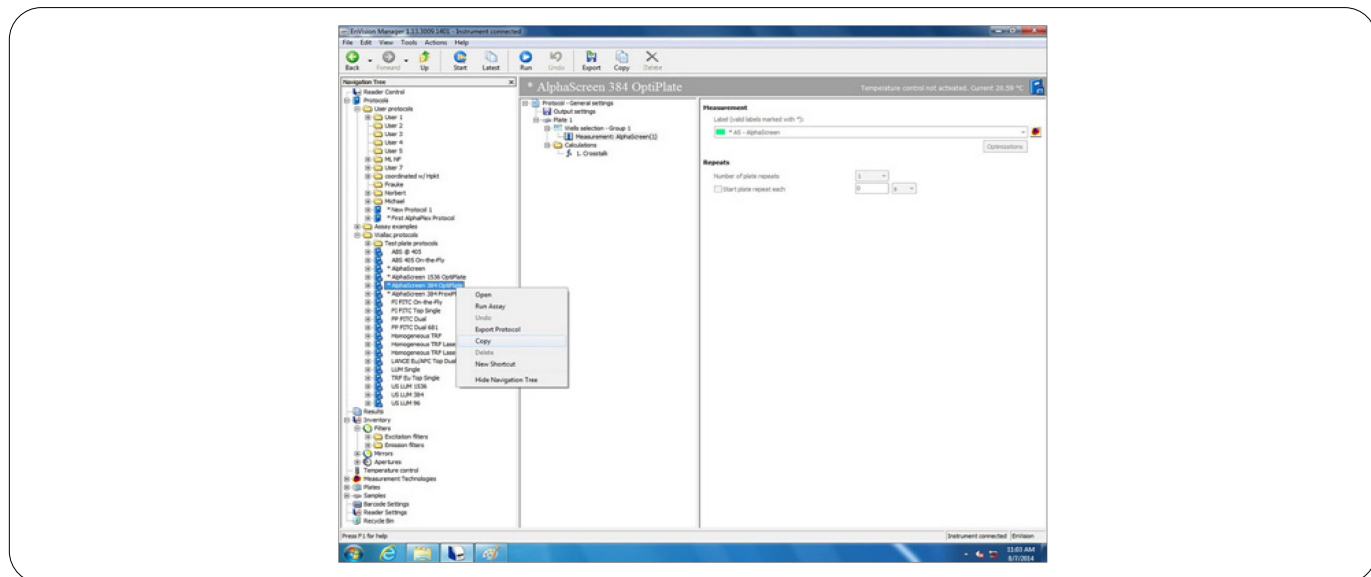
15. In the reader control right click "User protocols".
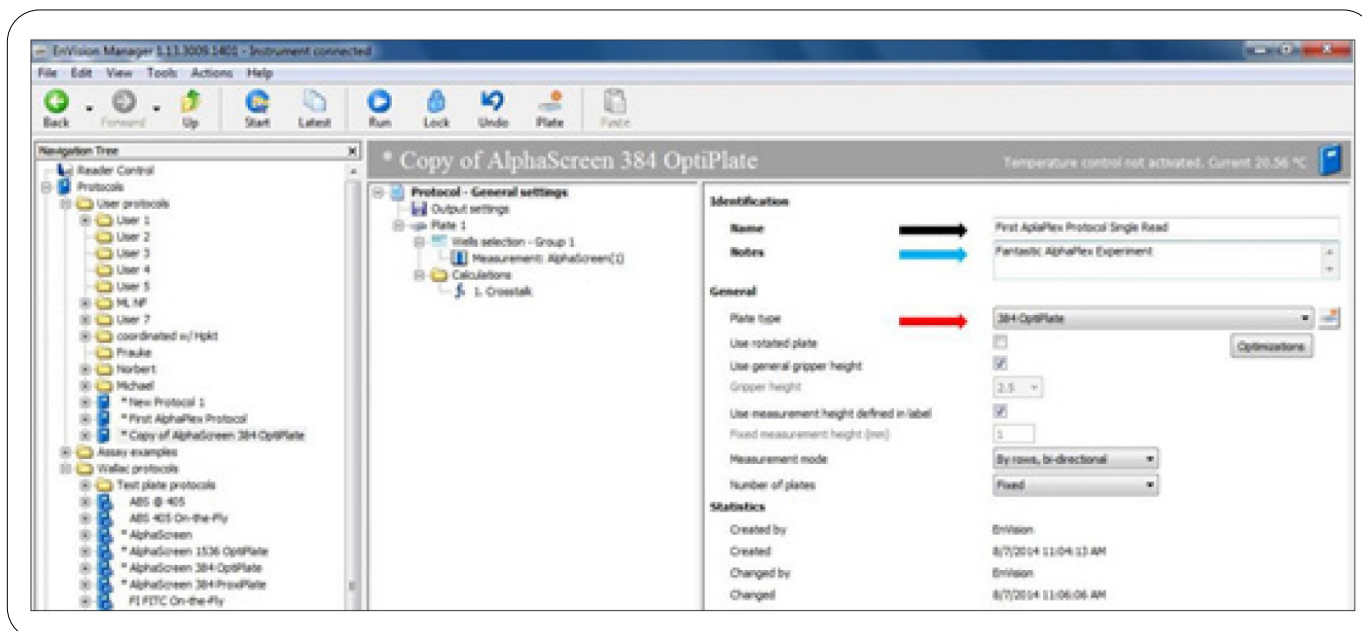
16. Select "Paste".

17. Double click on the new "Copy of AlphaScreen 384 OptiPlate" protocol.
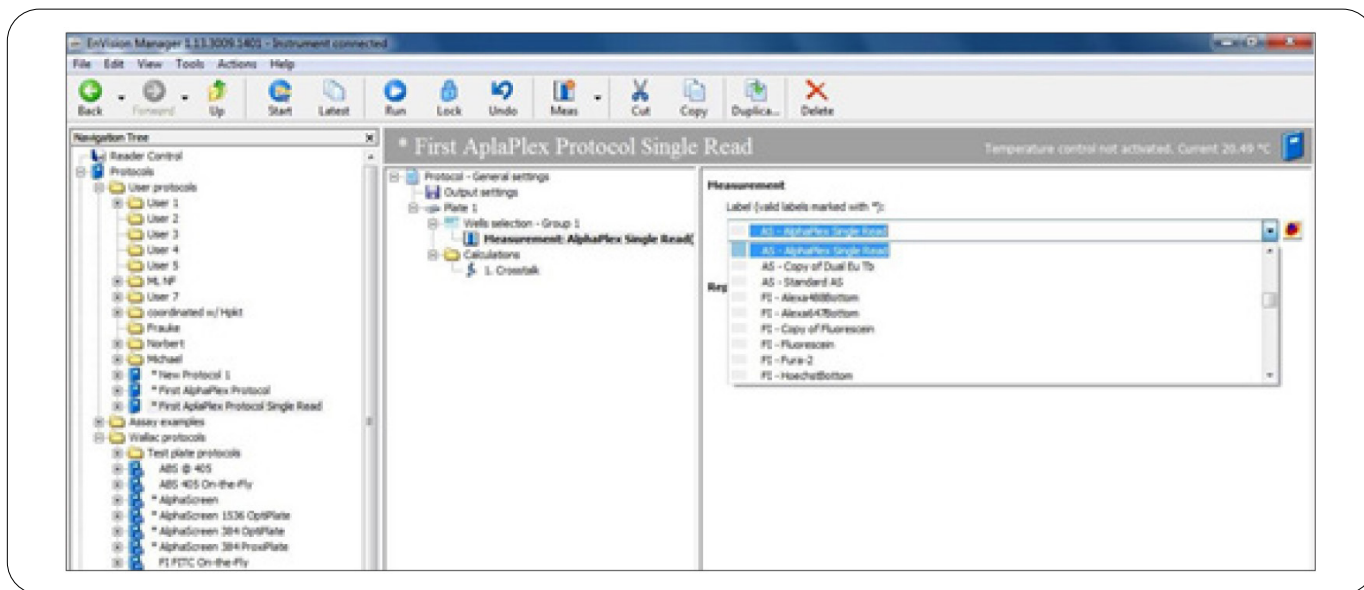
18. Click on "General Settings".

19. In the Name section give it the name you want. ▬▶

20. In the notes section note AlphaPlex single read. ▬▶

21. In the Plate section, select the type of plate you want to use. ▬▶

22. Click on "Measurement".

23. In the measurement label list select "AlphaPlex Single Read".

24. Click the "Back" button at the top of the window. The protocol will automatically save.

25. If all necessary filters are present, the new protocol name will be followed by an asterisk (*).