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Protocol for Labelling EV Samples with AcoDyes™ for Detection on Plate Reader

Table 1. Volume of solvent to add to obtain 25µM stock solution of AcoDyes™.

Packaging	Number of Tests	Volume of Solvent to Add	Solvent	Stock Concentration	Storage Conditions
0.5mL Vials	25 Tests (Trial Size)	25µL	Aqueous Buffer	25µM	2 to 8°C
	100 Tests	100µL			

1. To get a 25µM stock solution, reconstitute the dried dye in an aqueous buffer of your choice (e.g. sterile, filtered PBS). Refer to Table 1 above for the volume of solvent to add into the dye vial based on the product size (number of tests).
2. Vortex the dye for 1 minute to fully dissolve the solid.
3. Sonicate the dye for 15 minutes at 40°C.
4. Dyes perform best when freshly reconstituted. Solubilised dyes can be stored for up to 1 month at 4°C, but should be vortexed and sonicated for 15 minutes at 40°C before use.
5. **[Only for Aco-430™, Aco-490™, Aco-600™]** Aliquot a suitable volume of 25µM stock solution into a clean Eppendorf tube and dilute to 10µM using the same aqueous buffer.
6. Dilute EV sample to approximately **10^{10} particles/mL** using the same aqueous buffer.
7. In a 96-well clear polystyrene non-tissue culture treated plate, prepare the samples according to Table 2 below, mixing thoroughly. Avoid using the outer wells of the plate.

   **AcoDyes™ are provided in vials of 25µM stock concentration post-reconstitution. We recommend optimising staining for your sample by diluting the dye reagent in aqueous buffer and adding directly to your sample (final dye dilutions of 0.5µM, 1µM, 2.5µM, 5µM). Immediately post-addition, 1-2 hours incubation time is recommended at 37°C.*

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Table 2. Recommended EV sample preparation protocol. *Ensure that the ratio of dye particles to EV particles remains constant if you plan to deviate from the suggested volumes in Table 2.*

Samples to Run	Final Dye Conc* (µM)	Dye Solution		Volume of PBS* (µL)	Volume of 10^{10}/mL EV (µL)	Incubation
		Conc* (µM)	Volume* (µL)			
EV + Aco-430™	**0.5**	10	5	5	90	37°C for 1 hour, protected from light
EV + Aco-490™	**0.5**	10	5	5		
EV + Aco-600™	**1.0**	10	10	-		
EV + Aco-800™	**5.0**	25	20	-	80	37°C for 2 hours, protected from light
EV Only	**-**	-	-	10-20	80-90	Depends on AcoDye™
PBS Only	**-**	-	-	100	-	

8. Add to plate reader and collect full fluorescence spectrum or fluorescence at peak maxima.

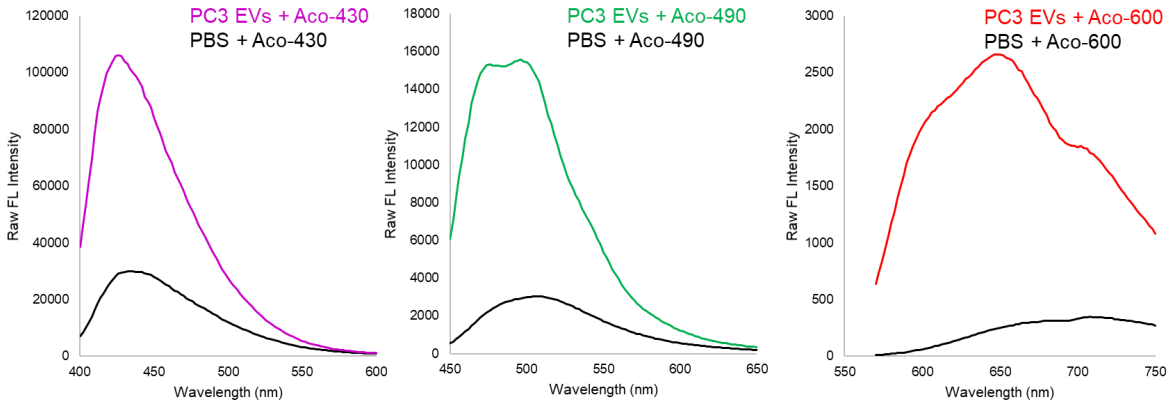

Figure 1. *PC3 Exosomes (Abcam) at approximately 10^9 particles/mL were mixed with 1µM of (left) Aco-430, (centre) Aco-490 or (right) Aco-600. Samples were incubated for 1 hour at 37°C before measuring on the Tecan Spark Microplate Reader. The samples were excited at their maximum absorption peak wavelength (refer to Table 3 below) and the full fluorescence spectra were collected.*

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Table 3. Photophysical properties of Aco-430™, Aco-490™, Aco-600™ and Aco-800™.

Dye Name	Aco-430	Aco-490	Aco-600	Aco-800
Appearance	Colourless	Yellow	Purple	Green
$\lambda_{abs,\ with\ SUVs}$ (nm)	369	422	525	735
$\lambda_{em,\ with\ SUVs}$ (nm)	403 - 460	458 - 508	586 - 635	775 - 818
Suitable Laser(s)	UV (355 nm)	Violet (405 nm)	Blue or Yellow (488 or 561 nm)	Red (638 nm)
Quantum Yield (%)	94	60	27	13.8
Fluorescence Lifetime (ns)	1.1	0.9	1.9	1.6
IC_{50} (μM)	>256	>256	170	>272

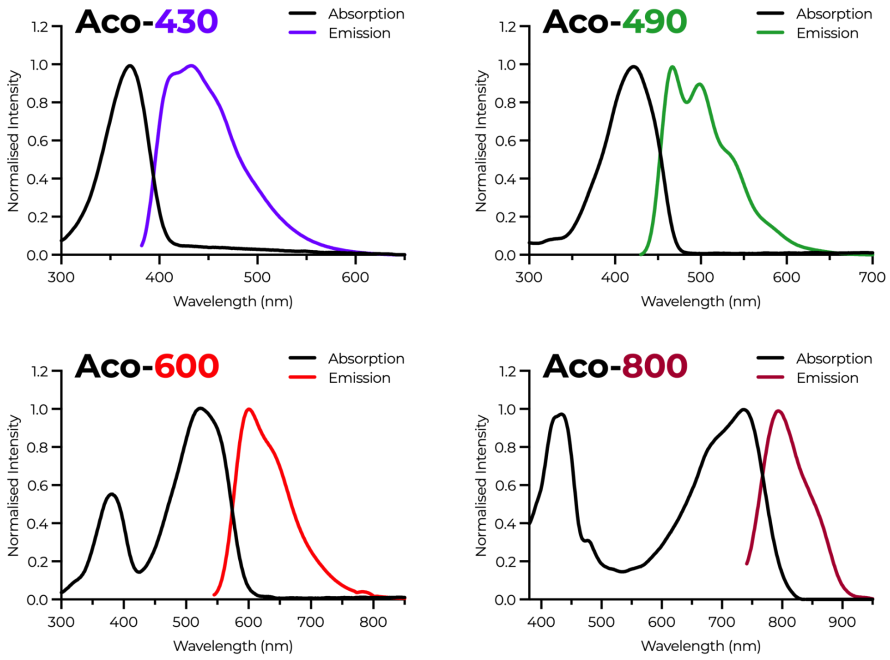

Figure 2. Absorption and emission spectra for Aco-430™, Aco-490™, Aco-600™ and Aco-800™.

Please feel free to email hello@acoerela.com for assistance with troubleshooting of the staining protocol.