Acoerela Pte. Ltd. | UEN: 202234149Z
Level 8, S9 Wet Science Building
National University of Singapore
4 Science Drive 2, Singapore 117544
hello@acoerela.com | www.acoerela.com

Protocol for Labelling EV Samples with AcoDyes™

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. Prepare the samples for flow cytometry according to Table 2 below:

   **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.*

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	1	1	18	37°C for 1 hour, protected from light
EV + Aco-490™	**0.5**	10	1	1		
EV + Aco-600™	**1.0**	10	2	0		
EV + Aco-800™	**5.0**	25	4	0	16	37°C for 2 hours, protected from light
EV Only	**-**	-	-	2-4	16-18	Depends on AcoDye™
Dye Only	**0.5 - 5.0**	10 / 25	1-4	16-19	-	

Acoerela Pte. Ltd. | UEN: 202234149Z
Level 8, S9 Wet Science Building
National University of Singapore
4 Science Drive 2, Singapore 117544
hello@acoerela.com | www.acoerela.com

8. **For detection on the <u>Beckman Coulter CytoFLEX LX Flow Cytometer</u>**, add 10µL of stained samples to 990µL of PBS just before analysis.

   The following data was obtained on the Beckman Coulter CytoFLEX LX Flow Cytometer using VSSC configuration. The exosomes used were purified using ultracentrifugation from the PC3 cell line commercially obtained from Abcam. Acoerela dyes for exosome detection have also been verified using the NanoFCM, Amnis ImageStream, BD FACSymphony A1, NovoCyte Penteon and the Thermo Fisher Attune.

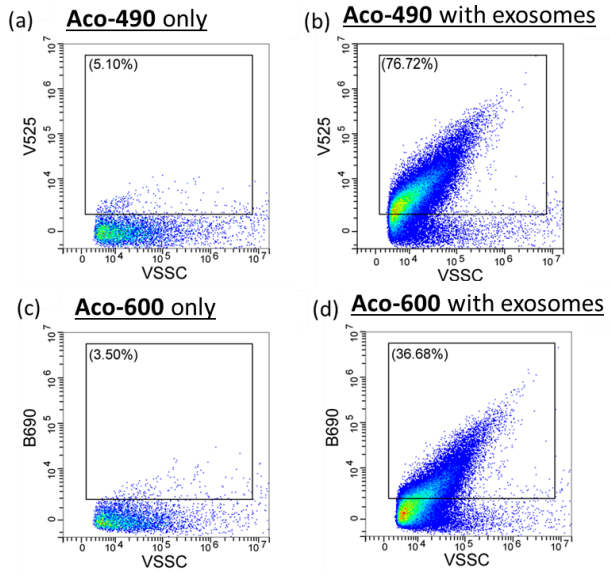

Figure 1. *Dot plot of events collected on the V525 channel (Excitation: 405nm, Emission: 525nm) for (a) Aco-490 only, (b) Aco-490 with exosomes, and on the B690 channel (Excitation: 488nm, Emission: 690nm) (c) Aco-600 only, (d) Aco-600 with exosomes.*

9. **For detection on the <u>NanoFCM Flow NanoAnalyzer</u>**, add 10µL of stained samples to 90µL of TAE or PBS just before analysis. *To combat stickiness of samples on the NanoAnalyzer, 1X TAE buffer can be used in place of PBS for resuspending EV samples and/or as the buffer used to dilute samples before running.*

   NOTE: Only Aco-600™ and Aco-800™ can be detected on the NanoFCM NanoAnalyzer.

   The following data was obtained on the NanoFCM Flow NanoAnalyzer machine. The samples used were Red Blood Cell Extracellular Vesicles (RBCEVs).

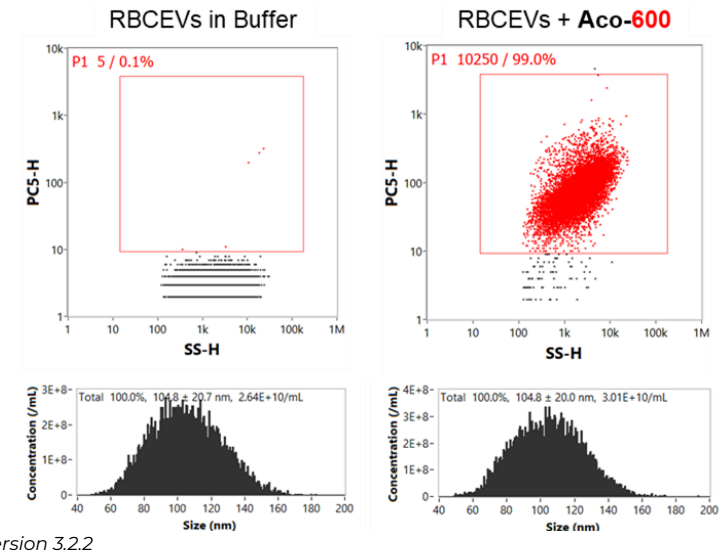

Figure 2. *Dot plot of events and size distribution (left) of RBCEVs only and (right) 0.1 µM* **Aco-600™** *+ RBCEVs (Excited using 488 nm blue laser, acquired using* ***613/18 nm filter****, on* ***PC5 channel****).*

For* **Aco-800™, *use the* ***783/56 nm filter*** *and acquire on the* ***APC750 channel****.*

Acoerela Pte. Ltd. | UEN: 202234149Z
Level 8, S9 Wet Science Building
National University of Singapore
4 Science Drive 2, Singapore 117544
hello@acoerela.com | www.acoerela.com

10. **For detection on the Cytek® Amnis® ImageStream®X Mk II Imaging Flow Cytometer**, add 10µL of stained samples to 250-500µL of PBS just before analysis.

    Instrument Settings for the ImageStream®X Mk II are suggested as follows:

    a. All lasers should be set to maximum power and data should be acquired with 60X magnification, a 7µm core size and low flow rate. Aco-430 emission can be collected on channel 1 (430-480 nm filter), Aco-490 on channel 2 (480-560nm filter), and Aco-600 on channel 3 (595-650nm filter). The brightfield channels can be set to any unused fluorescent channel for SSC detection. Speed beads can be left on to ensure proper sample focus.

    b. Further information on instrument settings and data analysis (gating strategy) can be found in the following publications:

    Front. Immunol. 9:1583.doi: 10.3389/fimmu.2018.01583
    Journal Of Extracellular Vesicles 2019, VOL. 8, 1587567

The following data was obtained on the ImageStream®X Mk II Imaging Flow Cytometer using 60X magnification.

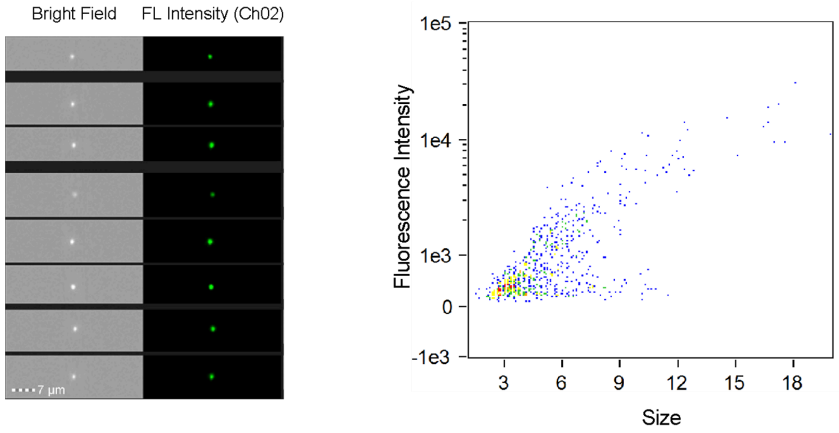

Figure 3. *PC3 Exosomes (Abcam) were stained with Aco-490. Samples were excited using a 405nm laser and emission was collected on Channel 2. Representative brightfield and fluorescence images (left) and dot plot of single exosomes (right) based on size and fluorescence intensity are as shown.*

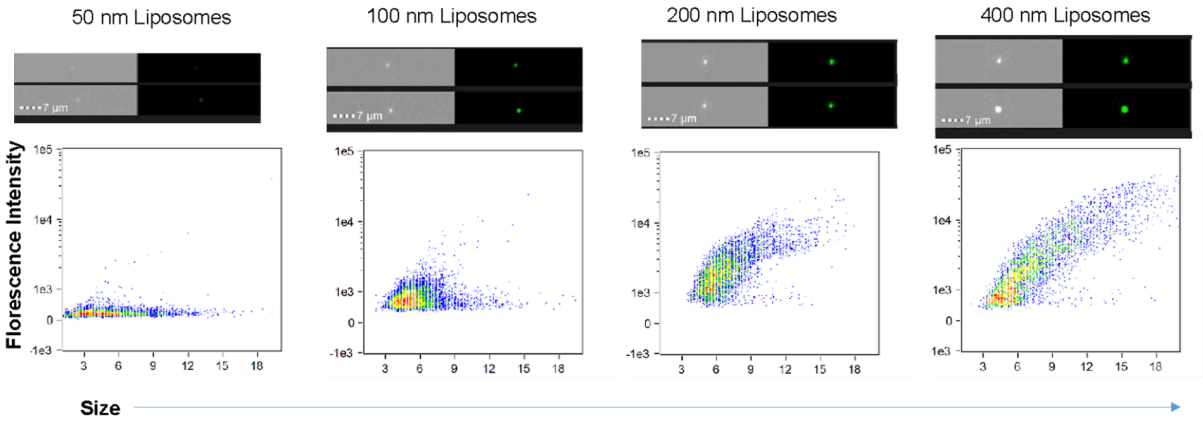

Figure 4. *POPC:POPG liposomes (4:1 molar ratio) were extruded to various sizes and stained with Aco-490. Samples were excited using a 405nm laser and emission was collected on Channel 2. Representative brightfield and fluorescence images (top) and dot plot of single liposomes (bottom) based on size and fluorescence intensity are as shown.*

Acoerela Pte. Ltd. | UEN: 202234149Z
Level 8, S9 Wet Science Building
National University of Singapore
4 Science Drive 2, Singapore 117544
hello@acoerela.com | www.acoerela.com

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

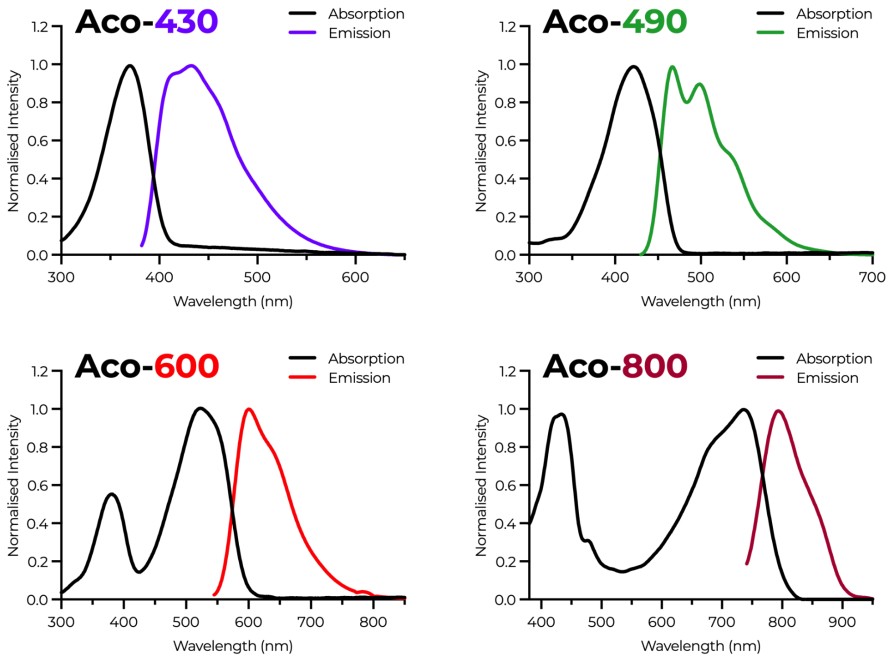

Figure 2. Absorption and emission spectra for Aco-430™, Aco-490™, Aco-600™ and Aco-800™.

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