Cell Reports Methods, Volume *2*

Supplemental information

MPA$_{PASS}$ software enables stitched multiplex, multidimensional EV repertoire analysis and a standard framework for reporting bead-based assays

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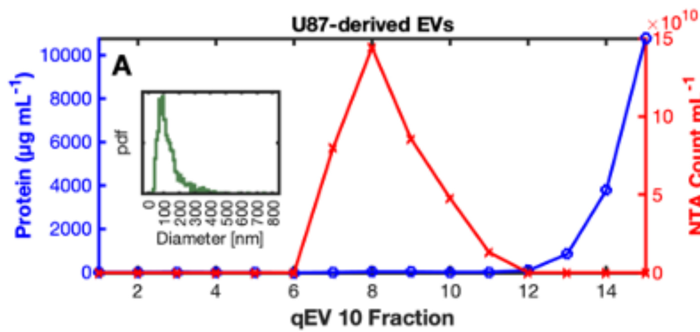
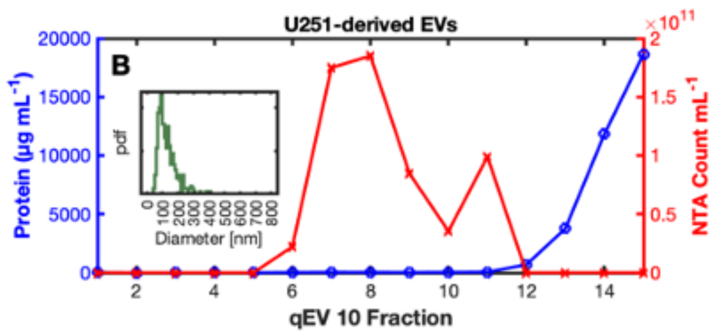
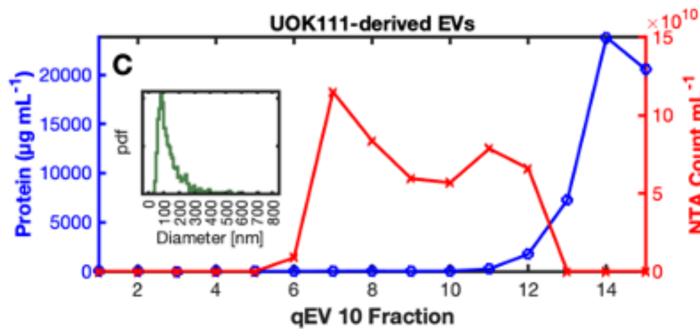
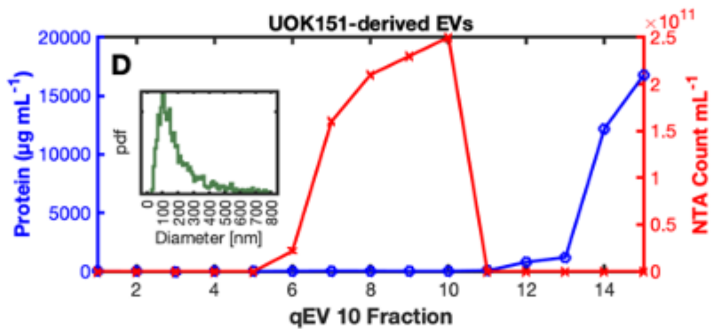
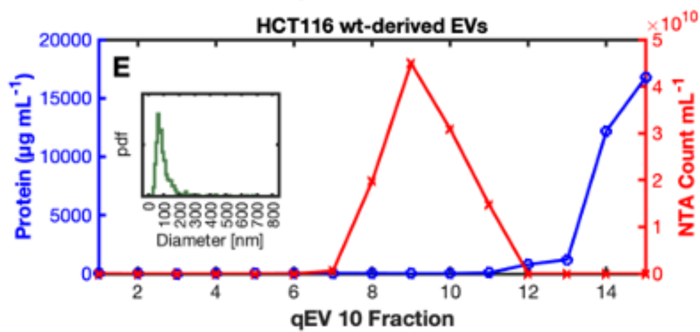
Figure S1. EV and protein concentration across qEV fractions. Overlaid on the elution fraction particle and protein concentration curves is a representative diameter distribution of each of the EV containing fractions used for the downstream multiplex analysis. Related to Figure 2 & 3.

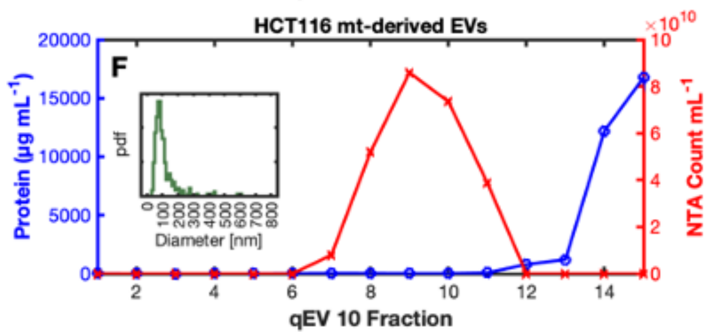

U87-derived EVs
A
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]

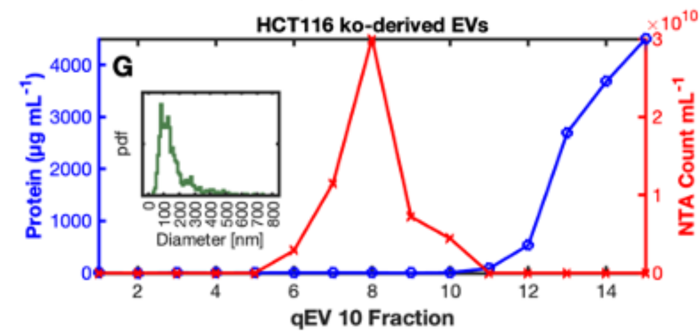

U251-derived EVs
B
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]


UOK111-derived EVs
C
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]


UOK151-derived EVs
D
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]


HCT116 wt-derived EVs
E
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]


HCT116 mt-derived EVs
F
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]


HCT116 ko-derived EVs
G
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]

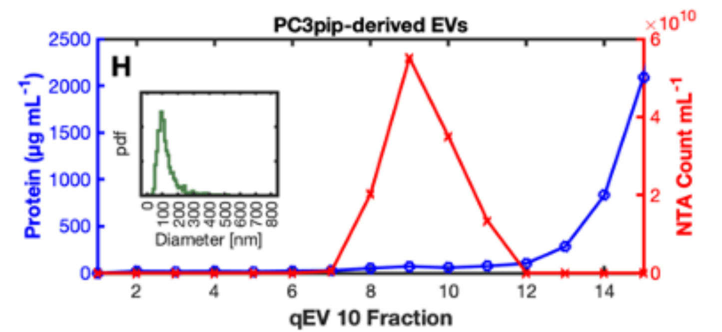
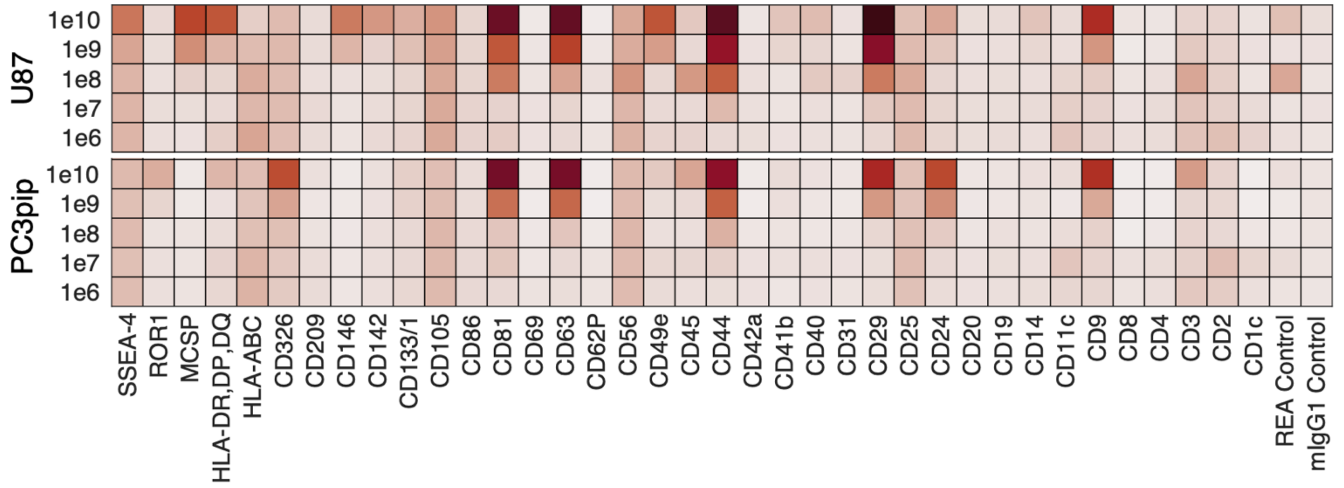

PC3pip-derived EVs
H
Protein (µg mL-1)
NTA Count mL-1
qEV 10 Fraction
pdf
Diameter [nm]

Figure S2. Multiplex input titration. Titration of two different cell line derived EVs from $1x10^6$ to $1x10^{10}$ total EVs. Scatter plots (bottom) showing the titration of total U87- and PC3pip-derived EVs when incubated with multiplex array. Intensities are of EV mix (CD9, CD63, CD81) and shown for a subset of capture beads (CD81, CD63, CD44, and CD29). Data was normalized using fold change. Related to Figure 2.


U87
PC3pip
1e10
1e9
1e8
1e7
1e6
SSEA-4
ROR1
MCSP
HLA-DR,DP,DQ
HLA-ABC
CD326
CD209
CD146
CD142
CD133/1
CD105
CD86
CD81
CD69
CD63
CD62P
CD56
CD49e
CD45
CD44
CD42a
CD41b
CD40
CD31
CD29
CD25
CD24
CD20
CD19
CD14
CD11c
CD9
CD8
CD4
CD3
CD2
CD1c
REA Control
mIgG1 Control

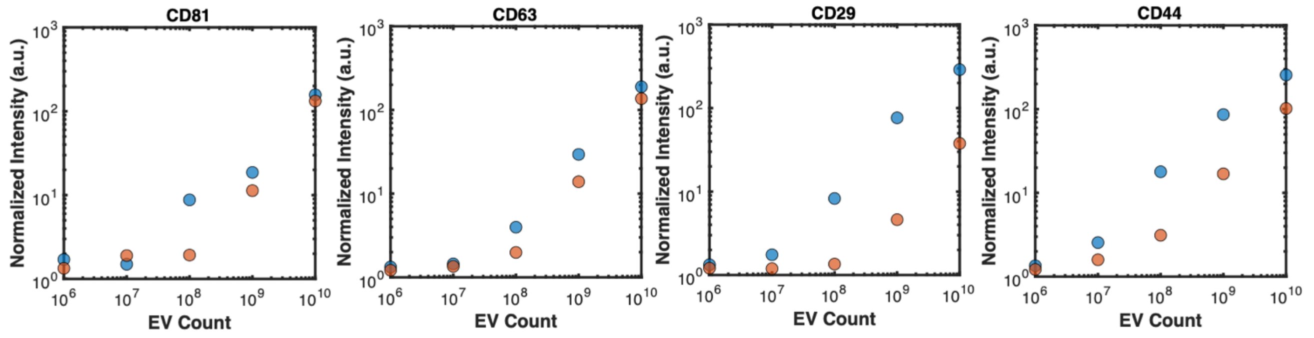
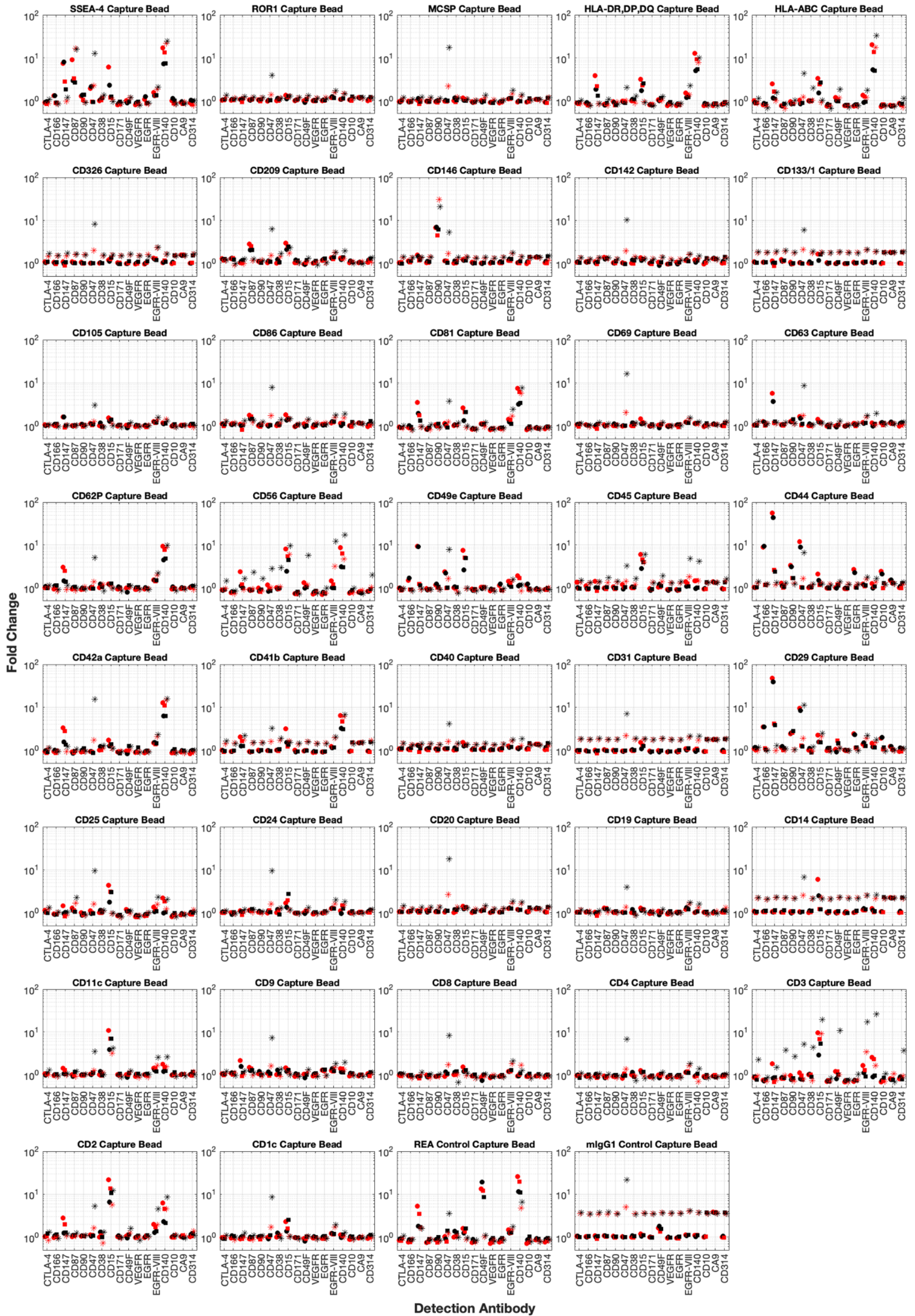
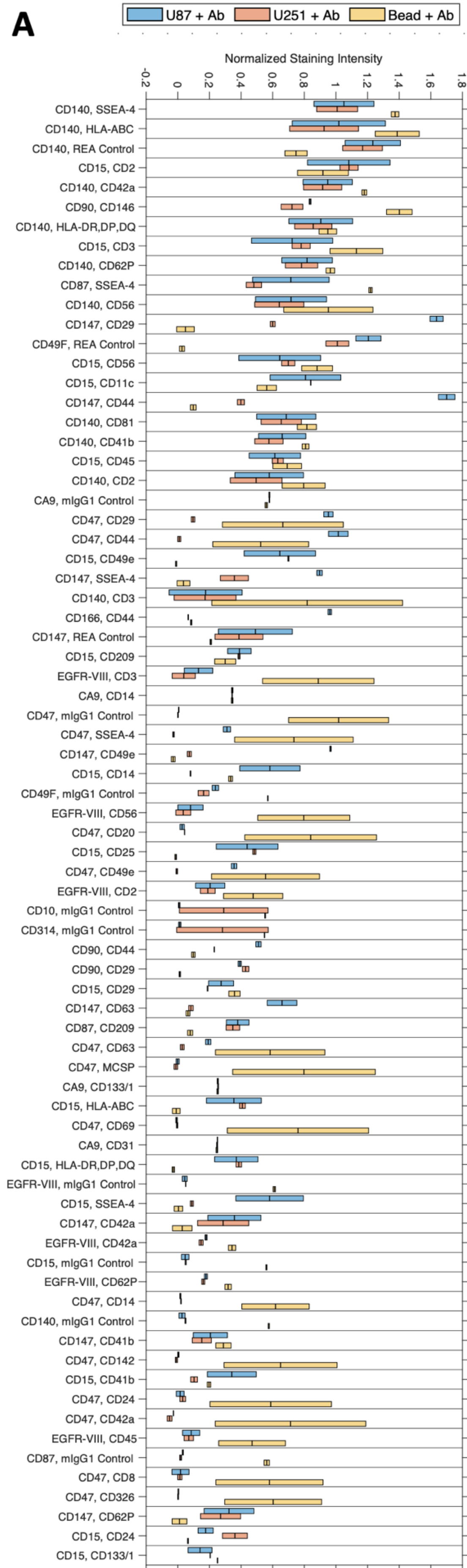

U87 EVs
PC3pip EVs
CD81
CD63
CD29
CD44
Normalized Intensity (a.u.)
EV Count

Figure S3. Screening antibodies for non-specific binding. All capture beads when incubated with 0.5 µg of detection antibody with buffer (asterixis markers) were incubated as negative controls. EV samples derived from U87 (circle marker) and U251 (square marker) cell lines were used as positive controls. The included Miltenyi buffer (red markers) and 2% EV-depleted FBS (black markers) were also compared. Related to Figure 3.

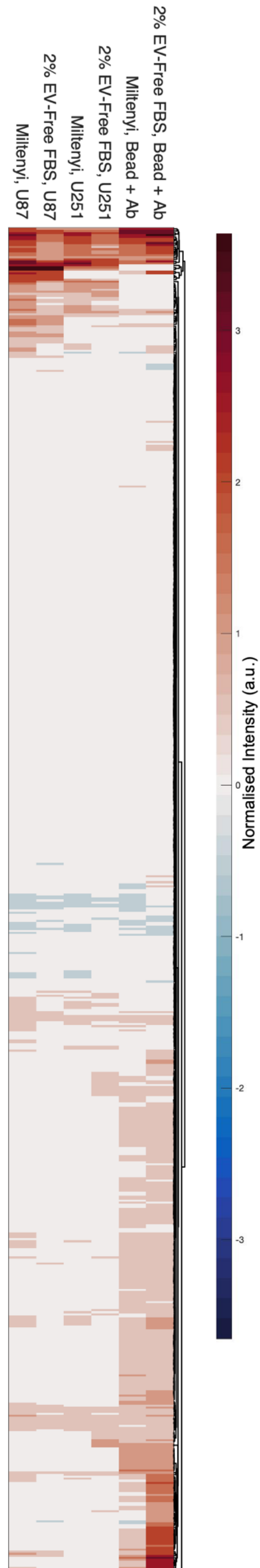


U87 EVs + Ab (Miltenyi)
U87 EVs + Ab (2% EVD FBS)
U251 EVs + Ab (Miltenyi)
U251 EVs + Ab (2% EVD FBS)
Bead + Ab (Miltenyi)
Bead + Ab (2% EVD FBS)
SSEA-4 Capture Bead
ROR1 Capture Bead
MCSP Capture Bead
HLA-DR,DP,DQ Capture Bead
HLA-ABC Capture Bead
CD326 Capture Bead
CD209 Capture Bead
CD146 Capture Bead
CD142 Capture Bead
CD133/1 Capture Bead
CD105 Capture Bead
CD86 Capture Bead
CD81 Capture Bead
CD69 Capture Bead
CD63 Capture Bead
CD62P Capture Bead
CD56 Capture Bead
CD49e Capture Bead
CD45 Capture Bead
CD44 Capture Bead
CD42a Capture Bead
CD41b Capture Bead
CD40 Capture Bead
CD31 Capture Bead
CD29 Capture Bead
CD25 Capture Bead
CD24 Capture Bead
CD20 Capture Bead
CD19 Capture Bead
CD14 Capture Bead
CD11c Capture Bead
CD9 Capture Bead
CD8 Capture Bead
CD4 Capture Bead
CD3 Capture Bead
CD2 Capture Bead
CD1c Capture Bead
REA Control Capture Bead
mIgG1 Control Capture Bead
10^{2}
10^{1}
10^{0}
CTLA-4
CD166
CD147
CD87
CD90
CD47
CD38
CD15
CD171
CD49F
VEGFR
EGFR
EGFR-VIII
CD140
CD10
CA9
CD314
Fold Change
Detection Antibody

Figure S4. Screening antibodies for non-specific binding using hierarchal clustering. A) Boxplot comparing the top 75 average bead intensities combinations across U87-derived EVs, U251-derived EVs, and bead + antibody controls. Phenotypes are order by detection antibody followed by capture antibody e.g. CD140 detection, SSEA-4 capture. **B)** heatmap showing hierarchically clustered raw data of U87-derived EVs, U251-derived EVs, and bead + antibody controls intensities. Related to Figure 3.



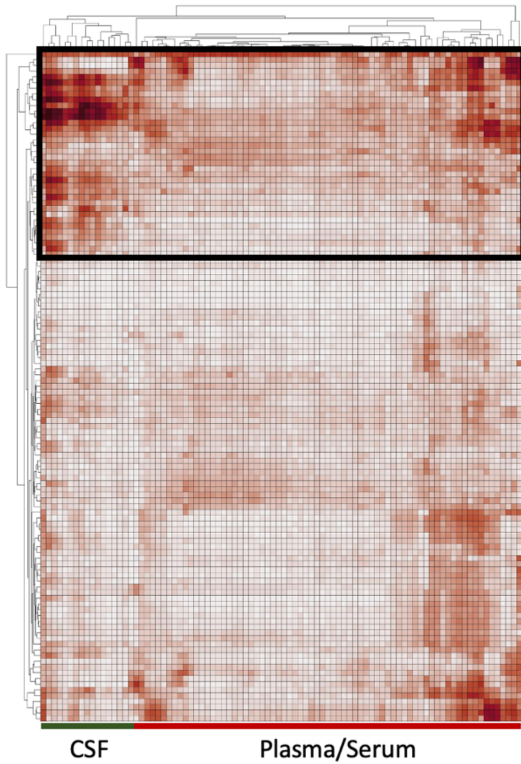
A
U87 + Ab
U251 + Ab
Bead + Ab
Normalized Staining Intensity
-0.2
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
CD140, SSEA-4
CD140, HLA-ABC
CD140, REA Control
CD15, CD2
CD140, CD42a
CD90, CD146
CD140, HLA-DR,DP,DQ
CD15, CD3
CD140, CD62P
CD87, SSEA-4
CD140, CD56
CD147, CD29
CD49F, REA Control
CD15, CD56
CD15, CD11c
CD147, CD44
CD140, CD81
CD140, CD41b
CD15, CD45
CD140, CD2
CA9, mIgG1 Control
CD47, CD29
CD47, CD44
CD15, CD49e
CD147, SSEA-4
CD140, CD3
CD166, CD44
CD147, REA Control
CD15, CD209
EGFR-VIII, CD3
CA9, CD14
CD47, mIgG1 Control
CD47, SSEA-4
CD147, CD49e
CD15, CD14
CD49F, mIgG1 Control
EGFR-VIII, CD56
CD47, CD20
CD15, CD25
CD47, CD49e
EGFR-VIII, CD2
CD10, mIgG1 Control
CD314, mIgG1 Control
CD90, CD44
CD90, CD29
CD15, CD29
CD147, CD63
CD87, CD209
CD47, CD63
CD47, MCSP
CA9, CD133/1
CD15, HLA-ABC
CD47, CD69
CA9, CD31
CD15, HLA-DR,DP,DQ
EGFR-VIII, mIgG1 Control
CD15, SSEA-4
CD147, CD42a
EGFR-VIII, CD42a
CD15, mIgG1 Control
EGFR-VIII, CD62P
CD47, CD14
CD140, mIgG1 Control
CD147, CD41b
CD47, CD142
CD15, CD41b
CD47, CD24
CD47, CD42a
EGFR-VIII, CD45
CD87, mIgG1 Control
CD47, CD8
CD47, CD326
CD147, CD62P
CD15, CD24
CD15, CD133/1



B
Miltenyi, U87
2% EV-Free FBS, U87
Miltenyi, U251
2% EV-Free FBS, U251
Miltenyi, Bead + Ab
2% EV-Free FBS, Bead + Ab
3
2
1
0
-1
-2
-3
Normalised Intensity (a.u.)

Figure S5. Identifying marker differences between biological fluids. A) Heatmap shows a high-level overview of hierarchically clustered samples and markers from samples of plasma, serum, and CSF stained when incubated with CD9, CD63, and CD81 independently across 39 unique capture beads and stitched analysis is applied. **B)** shows a selection of the heatmap where markers within CSF appear to be enriched when compared to plasma and serum. Related to Figure 4.

A



B

Normalised Intensity (a.u.)

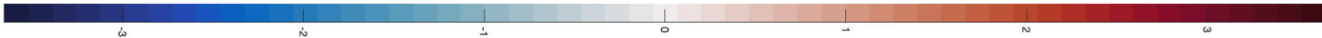


Figure S6 – Matched timepoint comparison of samples obtained from single individual with Streck, EDTA, Serum, Serum-Activated blood isolation tubes. Samples were incubated with a total count of $1x10^9$ or $1x10^{10}$ EVs either neat or post-qEV column. Due to it not being possible to count EVs from neat biological fluids without counting other particles, concentrations were assumed to be ~5-fold diluted by using the qEV column. Related to Figure 4.

A

1e9, Serum Activated, qEV, Fridge
1e9, EDTA, qEV, Frozen
1e9, EDTA, qEV, Fridge
1e9, Streck, qEV, Fridge
1e9, Serum, qEV, Frozen
1e9, Serum, qEV, Fridge
1e9, Streck, qEV, Frozen
1e10, Serum Activated, qEV, Frozen
1e10, Streck, qEV, Fridge
1e10, EDTA, qEV, Fridge
1e10, Streck, qEV, Frozen
1e10, EDTA, qEV, Frozen
1e10, Serum, qEV, Fridge
1e10, Serum, qEV, Frozen
1e10, Serum Activated, qEV, Fridge
1e9, Serum Activated, qEV, Frozen
1e9, EDTA, Neat, Frozen
1e9, Serum Activated, Neat, Fridge
1e9, Streck, Neat, Fridge
1e9, Serum Activated, Neat, Frozen
1e9, Streck, Neat, Frozen
1e9, Serum, Neat, Frozen
1e9, EDTA, Neat, Fridge
1e9, Serum, Neat, Fridge
1e10, Streck, Neat, Frozen
1e10, EDTA, Neat, Frozen
1e10, Serum Activated, Neat, Frozen
1e10, Serum, Neat, Frozen
1e10, Serum Activated, Neat, Fridge
1e10, Streck, Neat, Fridge
1e10, Serum, Neat, Fridge
1e10, EDTA, Neat, Fridge

EV Mix, CD41b
EV Mix, HLA-DR,DP,DQ
EV Mix, CD81
EV Mix, REA Control
EV Mix, CD42a
EV Mix, CD62P
EV Mix, CD9
EV Mix, CD63
EV Mix, CD29
EV Mix, CD69
EV Mix, MCSP
EV Mix, ROR1
EV Mix, CD20
EV Mix, CD142
EV Mix, CD24
EV Mix, CD105
EV Mix, CD25
EV Mix, CD326
EV Mix, CD133/1
EV Mix, CD86
EV Mix, CD19
EV Mix, CD209
EV Mix, mIgG1 Control
EV Mix, CD31
EV Mix, CD44
EV Mix, CD40
EV Mix, SSEA-4
EV Mix, HLA-ABC
EV Mix, CD56
EV Mix, CD14
EV Mix, CD45
EV Mix, CD3
EV Mix, CD2
EV Mix, CD1c
EV Mix, CD4
EV Mix, CD146
EV Mix, CD49e
EV Mix, CD8
EV Mix, CD11c

0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25

B

tSNE 2
tSNE 1
Neat
qEV

C

tSNE 2
tSNE 1
1e9
1e10

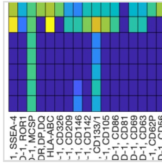
D

tSNE 2
tSNE 1
Fridge
Frozen

E

tSNE 2
tSNE 1
EDTA
Serum
Serum Activated
Streck

Supplementary Methods 1. Provides a detailed protocol and template for applying the multiplex samples to cell culture EVs that was used to generate data for Figure 1-3.



Sep 19, 2020

Multiplexed assay for detection of cell culture EV surface membrane proteins

In 2 collections

Joshua A Welsh[1], Bryce Killingsworth[1], Julia Kepley[1], Tim Traynor[1], Alexis Barfield[1], Jennifer Jones[1]

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1 Works for me Share

dx.doi.org/10.17504/protocols.io.be7yjhpw

Translational Nanobiology Section

Jennifer Jones

DISCLAIMER

This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

ABSTRACT

Protocol for using Miltenyi Biotec's human MACSplex Exosome Kit to assay one cell-line derived EV sample with up to 3 additional detection antibodies, at two EV count titration points (1E9 and 1E8 per LM10, NanoSight, NTA.). The optimisation of this protocol was done using bead kits released between 2017-2020.

DOI

dx.doi.org/10.17504/protocols.io.be7yjhpw

PROTOCOL CITATION

Joshua A Welsh, Bryce Killingsworth, Julia Kepley, Tim Traynor, Alexis Barfield, Jennifer Jones 2020. Multiplexed assay for detection of cell culture EV surface membrane proteins . **protocols.io** https://dx.doi.org/10.17504/protocols.io.be7yjhpw

COLLECTIONS

Stitched multiplexed assays allow multi-dimensional data analyses of EV repertoires.

2020 Featured Protocols

KEYWORDS

multiplex, flow cytometry, EVs

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CREATED

Apr 16, 2020

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LAST MODIFIED

Sep 19, 2020

OWNERSHIP HISTORY

Apr 16, 2020 Bryce Killingsworth

Aug 03, 2020 Jennifer Jones

PROTOCOL INTEGER ID

35800

PARENT PROTOCOLS

Part of collection

Stitched multiplexed assays allow multi-dimensional data analyses of EV repertoires.

2020 Featured Protocols

MATERIALS TEXT

MATERIALS

Low Protein Binding Collection Tubes (2.0 mL) **Thermo Fisher Catalog #88379**

MACSPlex Exosome Kit human **Miltenyi Biotec**

AcroPrep Advance Filter Plates for Aqueous Filtration - 350 µL 0.2 µm Supor membrane (10/pkg) **Contributed by users**

DISCLAIMER:

This protocol summarizes key steps for a specific type of assay, which is one of a collection of assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

BEFORE STARTING

- The protocol and attached planning template spreadsheet have been designed for an experiment assaying one cell-line derived EV sample with up to 3 additional detection antibodies, at two EV count titration points (1E9 and 1E8 per LM10, NanoSight, NTA.) Modifications of the spreadsheet and protocol may be necessary to use as a guide to assay multiple samples, human sample-derived EVs, or use additional numbers of detection antibodies.

Experiment planning

1 Determine which antibodies to use to detect EV surface membrane proteins in addition to the included CD9, CD63 and CD81 antibodies. All additional antibodies must be either APC or AF647 conjugated. Ensure you know the concentration of the antibodies, and if you are using an antibody conjugated in-lab, avoid preparations that have unbound dye.

2 Calculate the particle concentration of your EV sample, and the total particle count.

3 Use this template document to input your sample information and generate a plate map to visualize your experiment

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and the wells you will fill in the 96-well plate to be analyzed by the flow cytometer. Check the "How much volume of your sample is needed for this assay (µL)" section of the sheet to ensure your selections for EV count titration points are reasonable. If you have a very concentrated sample (~5E11part./mL or greater) you should increase the upper EV titration point to increase the fluorescent signal of the assay. If your sample is so dilute that you cannot incubate 1E9 EVs with each detection antibody consider methods to concentrate your sample as the fluorscent signal may be very weak and lower titration points not possible.

2020-07-30 - MACSplex Protocol Template.xltx

Additionally, this template was designed so that the user is directed to transfer a volume of EVs directly from the stock EV preparation into a tube with MACSplex capture beads and MACSplex buffer. However, for the lower titration point(s) the user may wish to prepare dilutions of their EV stocks in PBS and use equal volumes of EV dilutions for all mixes. For example Tube 1 contains 10 µL of a 1E11 part/mL EV dilution and Tube 2 contains 10 µL of a 1E10 part/mL EV dilution.

This template specifically applies to cell culture supernatant derived EVs. It is not recommended to use 10 µL (~15,000 beads) of beads with EVs derived from human fluids, such as plasma or serum as the bead recovery tends to be lower with biofluid ssamples than for cell culture supernatant EV preparations. A higher volume of beads would therefore be required.

4 Below is an example of how you might modify the template spreadsheet plate map to analyze more than one EV sample. Organize the plate so that the multichanel pipette can be used to transfer one antibody solution to a column or row. The "EV - bead capture" section of the spreadsheet will need to be duplicated and modified to help calculate needed volumes for each EV sample.

	1	2	3	4	5	6	7
A	CD9 1E9 EVs Cell line 1 10 µL capture beads	CD63 1E9 EVs Cell line 1 10 µL capture beads	CD81 1E9 EVs Cell line 1 10 µL capture beads	mAb 4 1E9 EVs Cell line 1 10 µL capture beads	mAb 5 1E9 EVs Cell line 1 10 µL capture beads	mAb 6 1E9 EVs Cell line 1 10 µL capture beads	Setup beads
B	CD9 1E8 EVs Cell line 1 10 µL capture beads	CD63 1E8 EVs Cell line 1 10 µL capture beads	CD81 1E8 EVs Cell line 1 10 µL capture beads	mAb 4 1E8 EVs Cell line 1 10 µL capture beads	mAb 5 1E8 EVs Cell line 1 10 µL capture beads	mAb 6 1E8 EVs Cell line 1 10 µL capture beads	5 µL capture beads
C	CD9 1E9 EVs Cell line 2 10 µL capture beads	CD63 1E9 EVs Cell line 2 10 µL capture beads	CD81 1E9 EVs Cell line 2 10 µL capture beads	mAb 4 1E9 EVs Cell line 2 10 µL capture beads	mAb 5 1E9 EVs Cell line 2 10 µL capture beads	mAb 6 1E9 EVs Cell line 2 10 µL capture beads	
D	CD9 1E8 EVs Cell line 2 10 µL capture beads	CD63 1E8 EVs Cell line 2 10 µL capture beads	CD81 1E8 EVs Cell line 2 10 µL capture beads	mAb 4 1E8 EVs Cell line 2 10 µL capture beads	mAb 5 1E8 EVs Cell line 2 10 µL capture beads	mAb 6 1E8 EVs Cell line 2 10 µL capture beads	
E	CD9 PBS control 10 µL capture beads	CD63 PBS control 10 µL capture beads	CD81 PBS control 10 µL capture beads	mAb 4 PBS control 10 µL capture beads	mAb 5 PBS control 10 µL capture beads	TGF-B1 PBS control 10 µL capture beads	

Citation: Joshua A Welsh, Bryce Killingsworth, Julia Kepley, Tim Traynor, Alexis Barfield, Jennifer Jones (09/19/2020). Multiplexed assay for detection of cell culture EV surface membrane proteinsÃÂ . https://dx.doi.org/10.17504/protocols.io.be7yjhpw

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Day 1: Incubating EVs with capture beads

5 Using the "EV - capture bead mix preparation" section of the template as a guide, determine the volumes of MACSplex buffer, MACSplex capture beads, and EVs that will be mixed together for the overnight bead capture incubation.

The spreadsheet has been designed so that the combined volume of MACSplex buffer and EVs in PBS is 65 µL for every 10 µL of MACSplex capture beads, or 75 µL of volume per detection antibody "test.'

6 Prepare an Eppendorf 2.0 mL LoBind collection tube for each EV sample being assayed, but before use ensure that the planned volume to be transferred to the tube is able to invert when the tube is rotated, otherwise the capture beads may sediment at the bottom of the tube during the overnight incubation and prevent proper interaction of EV proteins with their capture antibodies.

Other high quality low-protein binding tubes can be used if necessary.

7

Using our shared digital inventory, identify which capture bead tube you will use for this experiment and write down the lot number.

> CRITICAL STEP: The lot number is important for potential quality control issues that may arise with any of the 39 different bead populations.

8 Transfer the calculated amount of MACSplex buffer to each EV sample collection tube.

9 Spin down and vortex very thoroughly on the highest setting a tube of human MACSPlex Exosome Capture Beads and aliquot the calculated volumes into each prepared EV sample collection tube.

10 Vortex and then add the calculated volume of undiluted or diluted EVs to each labeled tube containing MACSplex buffer and capture beads.

11

Vortex each tube well, then place in a tube rotator, covered with foil, and rotate overnight at RT.

> CRITICAL STEP: make sure the volume inside each tube is inverting and falling down the side of the tube when it is rotated so that the large capture beads remain in suspension throughout the night to allow for adequate mixing.

Day 2: Staining captured EVs with detection antibodies

12 Use the "Antibody preparation table" section of the template to calculate the volumes of antibodies and buffer you will need to prepare for the detection antibody staining step of the assay.

Citation: Joshua A Welsh, Bryce Killingsworth, Julia Kepley, Tim Traynor, Alexis Barfield, Jennifer Jones (09/19/2020). Multiplexed assay for detection of cell culture EV surface membrane proteinsÃÂ . https://dx.doi.org/10.17504/protocols.io.be7yjhpw

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13 Get 1 new Pall 0.2 µm PES filter plate.

14 Using a multichannel pipet, add 150 µL of MACSplex buffer to all sample and control wells.

It is recommended to use the top portion of the vacuum manifold as a plate holder to aid pipetting.

Screen Shot 2020-07-30 at 10.29.13 PM.png

Figure 1: Pall Vacuum Manifold (ID: 5017.) The silver metal top is removable and rests on the blue metal bottom component. The blue pressure valve can be opened slowly to prevent sudden pressure changes disturbing fluid. Vacuum should only be applied until sufficient to empty wells of fluid. The silver metal top can be used as a temporary plate rack for pipetting and mixing samples during washing steps. Figure from Pall's website.

15 Subject the plate to vacuum just until all wells are emptied of buffer.

(release the vacuum pressure gently by pressing the vacuum release rapidly)

Quickly and gently blot the bottom of the filter plate against a clean paper towel.

16 As quickly as possible, add 50 µL of MACSplex buffer to previously wetted wells.

17 Vortex each EV sample tube very thoroughly and add 75 µL to each test well.

75 µL should contain 10 µL of capture beads with EVs bound to them.

Based on the template spreadsheet you should have 10% excess volume so there should be no concern with running out of sample for the last detection well on the filter plate.

(To aspirate all volume at the bottom of the collection tube, the tube may need to be spun down using a bench-top centrifuge and then quickly vortexed or pipetted again.)

18 Add 10 µL of vortexed MACSplex capture beads to the indicated detection antibody control wells (1 per detection antibody.)

19 All detection antibodies should be vortexed gently and spun quickly in a table top centrifuge if possible.

20 Prepare all antibody solutions according to the template spreadsheet. If doing a large experiment make sure to only prepare the antibody solutions immediatley before use.

21 Once antibody solutions are prepared, vacuum the plate until the wells are just empty of liquid. Very quickly and gently blot the bottom of the filter plate against a clean paper towel to remove any drops of buffer on the bottom of the plate.

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22

Using a multichanel pipette and reagent well if possible, and dispensing quickly to prevent filter plate membranes from drying out, dispense antibody solutions onto their indicated wells, 200 µL of antibody solution per well. Avoid bubble formation and do not mix initially.

CRITICAL STEP: when using the multichanel pipette it is easy to accidentally draw up unequal volumes of solution. Rock the pipette very firmly on the tips to ensure a strong connection, ensure all tips are level by visual inspection before use, when drawing up fluid ensure all tips are fully placed below the top level of the reagent, and **visually confirm** that each tip has a similar amount of fluid. All the fluid should remain at the bottom of the pipette tip and not drift higher up in the tip, otherwise it will not be dispensed equally and bubbles will form in an attempt to eject residual fluid.

23 Using a multichannel pipet set to 100 µL, mix the volumes in the wells up and down, without contacting the filter membranes at the bottom of the wells to avoid puncturing them. The tip should initially be placed exactly in the middle of the fluid in the wells, then rotated slightly toward the sides of the well to mix beads which may be on the side of the well.

Screen Shot 2020-07-30 at 10.48.28 PM.png

Figure 2: Three ~400 µL wells with a 0.2 µm membrane at the bottom. The triangle represents the optimal placement of the pipette tip during mixing. One scrape of the membrane does not mean the experiment is ruined, but hard scrapes can either puncture a hole in the membrane, allowing bead-captured EVs to spill out, or introduce membrane debris into the sample mixture.

24 Cover the plate with a foil plate sealer and incubate for 2 hours at RT, shaking.

25 After 2 hours, vacuum the plate until fluid just empty, and then add 150 µL of MACSplex buffer to all used wells immediately.

26 Using the 75 µL setting, with the plate positioned on top of the silver metal part of the manifold, reverse pipette all wells with careful tip positioning to avoid bubbles but also not scrape filter membrane.

27 Clear all wells with the vacuum.

28 Immediately added 150 µL buffer to all wells

29 Using the 75 µL setting, with plate positioned on top of manifold, reverse pipette all wells with careful tip positioning to avoid bubbles but also not scrape filter membrane

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30 Place on vacuum manifold until all wells are cleared

31 Immediately add 200 µL of buffer to all wells.

This final resuspension volume can be adjusted to produce more or less concentrated final suspensions of the capture beads, depending on the optimal concentration to run samples on the flow cytometer.

32 Using 200 µL setting, with plate on top of metal manifold piece, all wells should be reverse pipetted with tip positioning careful to avoid bubbles but also not scrape filter membrane and then transferred to Axygen racked mini 1.1 mL tubes, or a 96-well plate compatable with the plate reader for the particular clow cytometer being used.

When preparing this final resuspension press the mutlichanel plunger half-way to its stopping point to use a volume of ~100 µL to fully resuspend all capture beads sitting on top of the filter plate membrane.

33 Add 5 µL of capture beads in 200 µL of PBS to a free well on the plate or tube rack for the cytometer, as well as 20 µL of setup beads with 20 µL of PBS (varies largely depending on setup bead concentration) to another free well.

Day 2: running plate on cytometer

34 Run setup beads on the flow cytometer to adjust cytometer settings and gate around a single bead population.

35 Analyze the full volume of each 200 µL stained EV sample, including the non-EV containing capture bead + detection antibody controls to check for nonspecific binding of detection antibody to capture beads.

Data analysis

36 Use MPA_{PASS} software to analyze the multiplexed EV protein expression data. Protocol under development.

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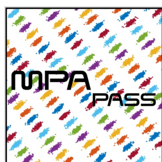
Supplementary Methods 2. Provides a detailed protocol for bead gating strategies using FlowJo Software that was used to generate data for Figure 1-5.



bu3mnyk6

MPAPASS - Gating flow cytometry multiplex data V.(bu3mnyk6)

Joshua A Welsh[1], Sean M Cook[1], Jennifer Jones[1]

[1]Translational Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

1 Works for me

Joshua Welsh

DISCLAIMER

This protocol summarizes key steps for a specific type of method, which is one of a collection of methods and assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

ABSTRACT

This is collection contains the protocols required for each step in the mpapass software pipeline for performing stitched multiplex analysis. This is one of a number of protocols in the pipeline for using the mpapass software package and is applicable to the latest release of the software.

PROTOCOL INFO

Joshua A Welsh, Sean M Cook, Jennifer Jones . MPAPASS - Gating flow cytometry multiplex data. **protocols.io**
https://protocols.io/view/mpapass-gating-flow-cytometry-multiplex-data-bu3mnyk6

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May 17, 2021

LAST MODIFIED

May 17, 2021

PROTOCOL INTEGER ID

49997

MATERIALS TEXT

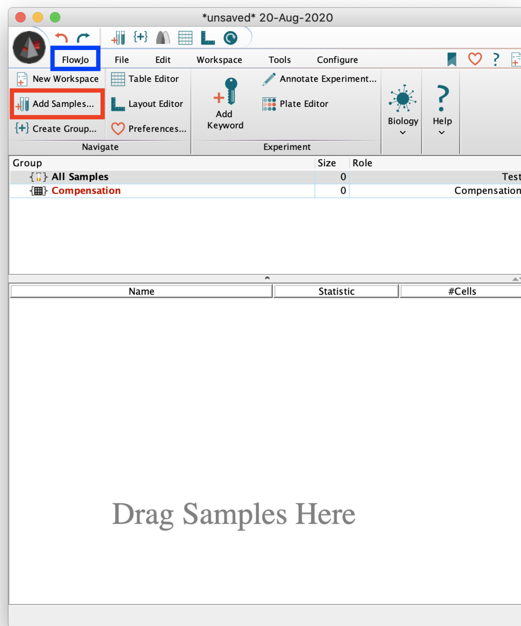
FlowJo

DISCLAIMER:

This protocol summarizes key steps for a specific type of method, which is one of a collection of methods and assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

Importing the Files into FlowJo

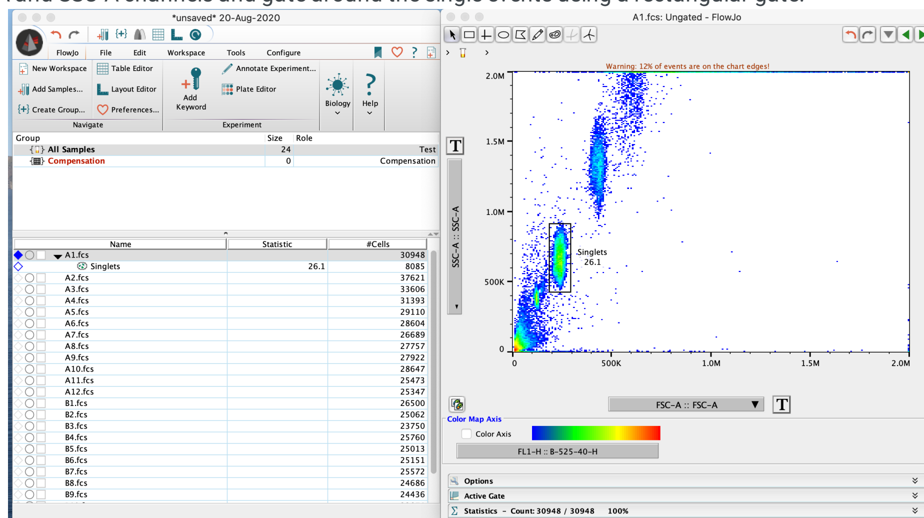
1. Import the desired files into the FlowJo workspace using either the Add Samples (red box) button under the FlowJo tab (blue box), or simply drag the desired files into FlowJo.



It is recommended that the fluorescence parameters be calibrated into standard units of 'molecules of equivalent soluble fluorophore' (MESF) to aid in reproducibility of data. This can be done using FCMPASS software in combination with commercially available MESF beads. See the FCMPASS software for further information at https://nano.ccr.cancer.gov/fcmpass

Gating the Bead Populations

2 Double-click on any of the samples in order to bring up a scatter plot. Change the parameters of the scatter plot to FSC-A and SSC-A channels and gate around the single events using a rectangular gate.



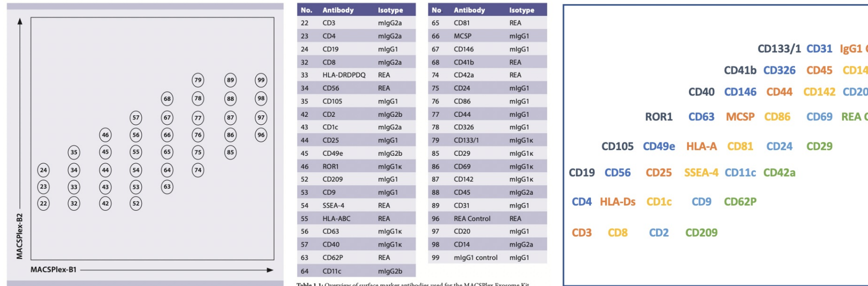
In this example, the A1.fcs sample was used to gate for singlets.

3 Double-click on the newly gated Singlets population to open a new scatter plot. Change the parameters to the FITC-A and PE-A channels, and a pattern similar to the figure below should be seen.

There are supposed to be 39 distinct bead populations, however in kits manufactured between 2018-2019, only 38 can be seen due to the merging of the 89 and 99 bead populations.

image.png

4 Each of the distinct populations corresponds to a antibody coated bead that can be determined from the bead legend below:



No.	Antibody	Isotype	No	Antibody	Isotype
22	CD3	mIgG2a	65	CD81	REA
23	CD4	mIgG2a	66	MCSP	mIgG1
24	CD19	mIgG1	67	CD146	mIgG1
32	CD8	mIgG2a	68	CD41b	REA
33	HLA-DRDPDQ	REA	74	CD42a	REA
34	CD56	REA	75	CD24	mIgG1
35	CD105	mIgG1	76	CD86	mIgG1
42	CD2	mIgG2b	77	CD44	mIgG1
43	CD1c	mIgG2a	78	CD326	mIgG1
44	CD25	mIgG1	79	CD133/1	mIgG1κ
45	CD49e	mIgG2b	85	CD29	mIgG1κ
46	ROR1	mIgG1κ	86	CD69	mIgG1κ
52	CD209	mIgG1	87	CD142	mIgG1κ
53	CD9	mIgG1	88	CD45	mIgG2a
54	SSEA-4	REA	89	CD31	mIgG1
55	HLA-ABC	REA	96	REA Control	REA
56	CD63	mIgG1κ	97	CD20	mIgG1
57	CD40	mIgG1κ	98	CD14	mIgG2a
63	CD62P	REA	99	mIgG1 control	mIgG1
64	CD11c	mIgG2b			

Table 1.1: Overview of surface marker antibodies used for the MACSPlex Exosome Kit.

5 Gate each of the bead populations using a elliptical gate and label the populations according to the bead legend in step 4. For the merged population, gate around the entire population--later the population wil be separated.

image.png

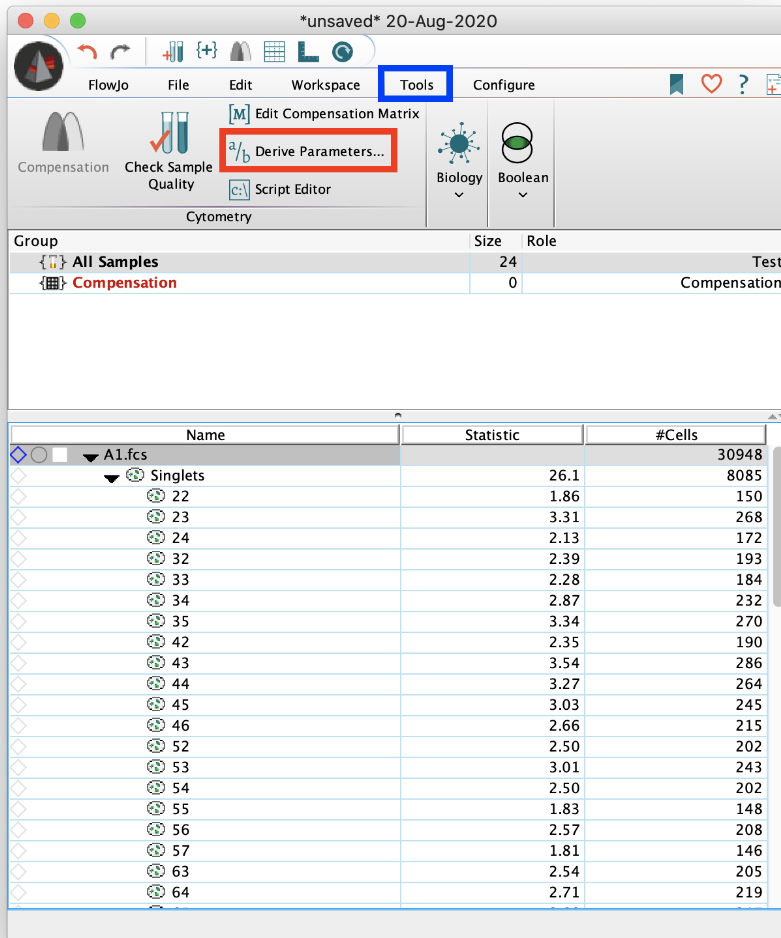
Separating the Merged Population

6 On select cytometers it has been found that some lots of Miltenyi exosome multilpex beads do not adequately separate populations 89 and 99. If this is not the case proceed to the next section.

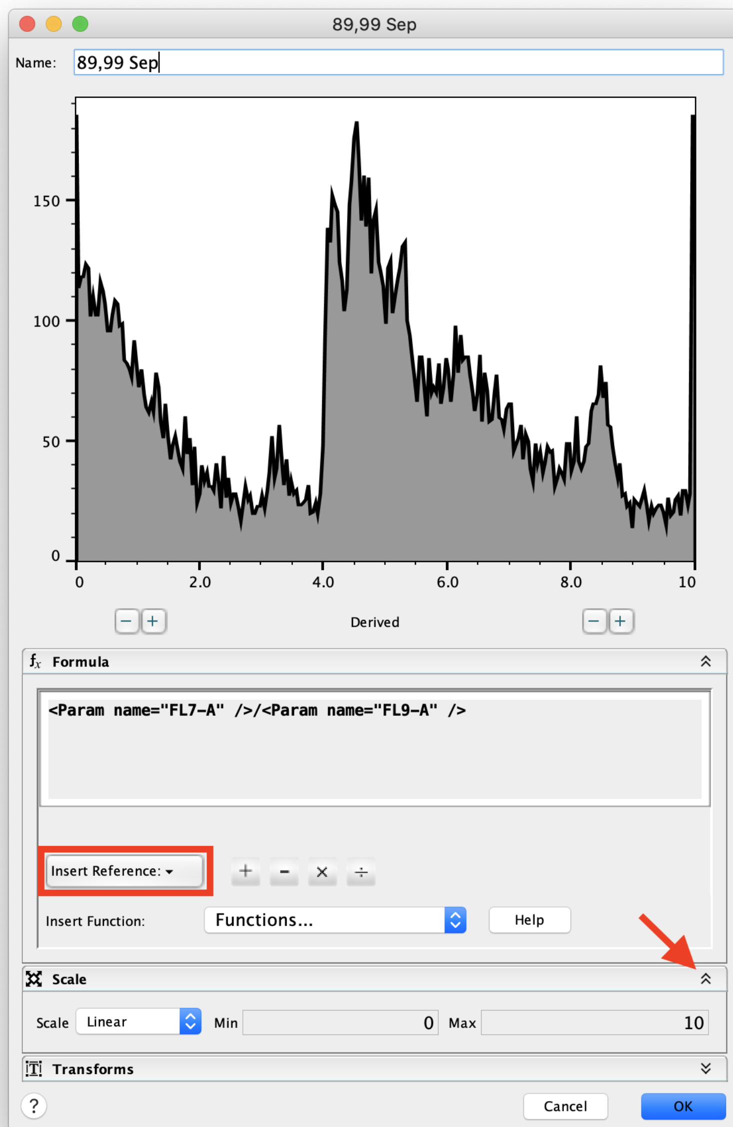
The FlowJo workspace should now have subpopulations under the Singlet population for each bead population as well as the merged population.

Click on the sample used to gate the bead populations (the highlighted A1.fcs sample in the figure below) and then navigate to the tools tab (blue box).

Click on Derive Parameters (red box) which will bring up a new window.



7 Creating the Derived Parameter:



7.1 In this new window, at the top, name the derived parameter 89,99 Sep.

7.2 Using the Insert Reference button (red box), choose the V-525-A channel. A string of characters will appear in the text box above.

Click on the division button next to the Insert Reference button.

Once again using the Insert Reference button (red box), now choose the V-660-A channel.

A plot similar to the figure above should be now be seen

7.3 Click on the collapse/open arrows for the Scale tab (denoted by a red arrow).

Choose the Linear scale and set the Min to 0 and the Max to 10.

Now click OK, and the derived parameter should appear beneath the chosen sample.

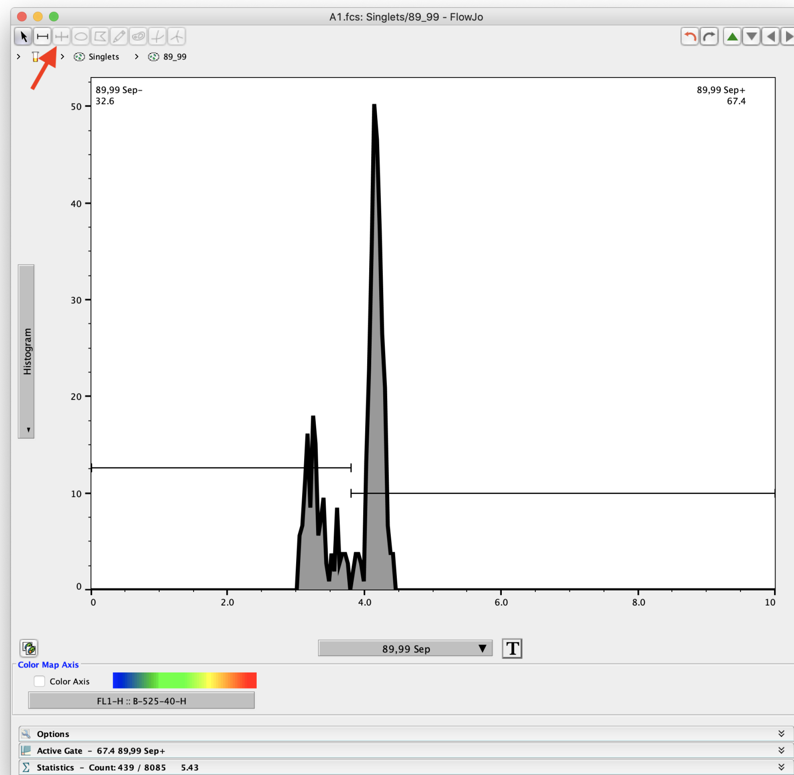
8 **Gating the Merged Population:**

8.1 Click on the 89_99 subpopulation to open a new scatter plot.

Choose 89,99 Sep derived parameter from the dropdown menu on the X-axis and the histogram option on the Y-axis.

A plot similar to the figure below should be seen.

8.2 Click on the Bisector Tool (red arrow) to gate the two bead populations. Make sure to choose a point where the two populations are distinctly separated.



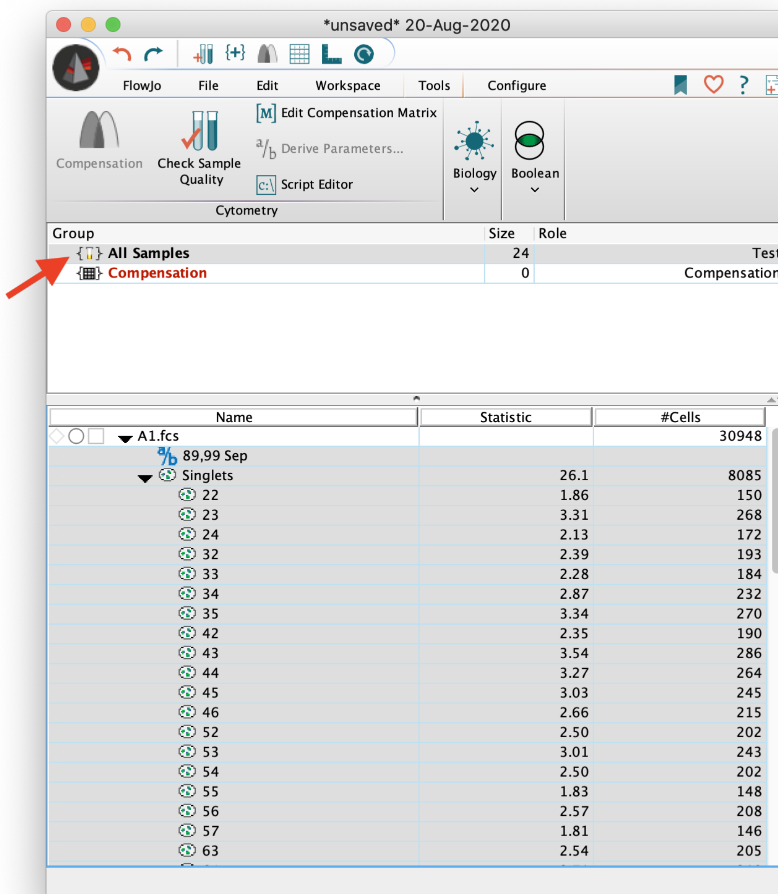
8.3 The populations should be relabeled by right clicking the populations in the FlowJo workspace. The upper population should be labeled 99 while the lower population should be labeled 89.

Applying the Gates to Each Sample

9 In the FlowJo workspace, highlight all the gated populations and the derived parameter.

Now drag them into All Samples Group above (red arrow).

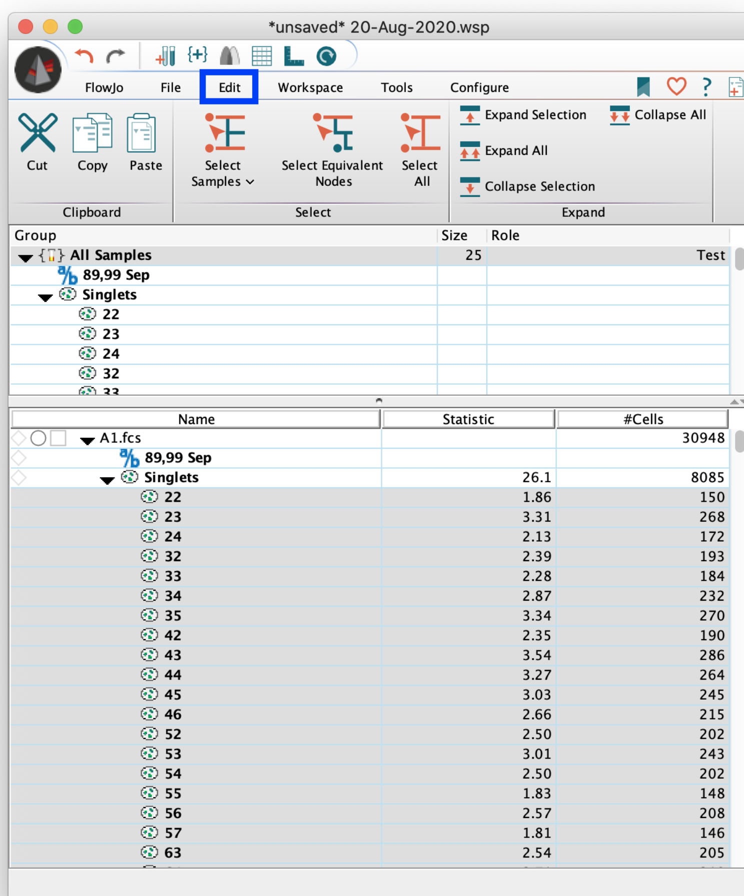
All files in the FlowJo workspace should now be gated exactly how we gated our chosen sample. This will be reflected in the FlowJo workspace as all samples should now have the same populations.



Exporting the Files

10 Now that all our samples have been gated, it is time to export the data from FlowJo into .csv files.

For any of the samples select all the bead populations to export, then under the Edit tab (blue box), click the Select Equivalent Nodes button to select all bead populations from every sample to export.



11 Navigate to the file tab and click on the Export/Concatenate Populations button.

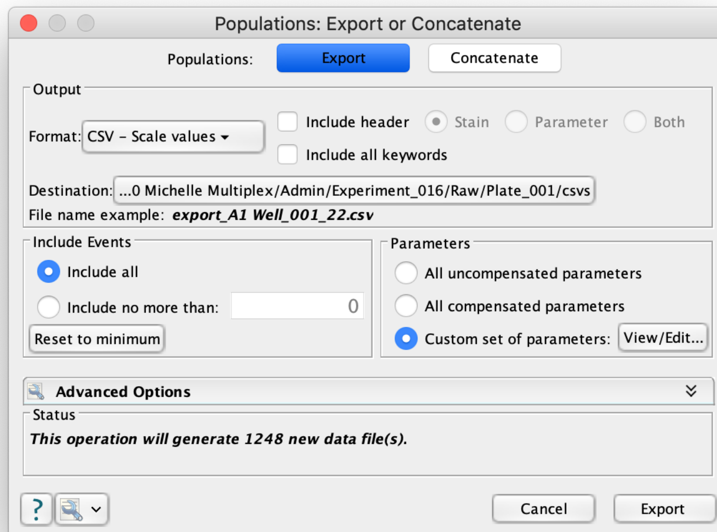
12 Change the format to CSV - Scale Values.

Uncheck 'Include Header'

Change the Destination to desired folder.

Click on Custom Set of Parameters and then the View/Edit button directly next to it. Choose the APC parameter from the pop-up list.

Finally click Export to generate the .csv files in the destination folder.



13 In preparation for the MPAPASS software, you will have to manually rename the 99 bead population from the export suffix of 89_99_99.csv to simply _99.csv

Repeat if this occurs with the 89 bead population.