EVix Single-Tope Kit

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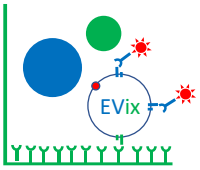


The EVix Single-Tope ELISA kit captures EVs with 115 unique capture antibodies coated in individual wells. The captured EVs are detected with user selected biotinylated antibodies. Each kit contains 115 different antibody specific capture wells in duplicate, including CD9, CD63 and CD81 in multiple wells for a total of 256 individual assay wells. Duplicate capture wells are run with and without EVs providing negative controls for each capture and detection antibody combination .

Use to phenotype EV preparations by antigen content. Single-Tope kits are used to characterize EV preparations from different sources or production runs. A detailed finger print of antigen presence is obtained. Data from the Multi-Tope assay can be further examined using alternative detection antibodies in the Single-Tope kit. As example, one can search for antigen combinations which may define unique EV populations within a preparation. The pattern of expression of the selected detection antibody targeted antigen, can be evaluated on EVs captured by 115 different capture antibodies and compared to patterns from other EV preparations.

Kit Contents: If contents are unopened and stored at 4° C the performance of the kit is stable for 6 months.

A: Single Tope Capture Plates: Three (MT1, MT2 and MT3) 96 well plates containing 256 individual assay wells. These are the same plates used in the Multi-Tope assay and Single-Tope data can be compared to results obtained in the Multi-Tope assay.

B: 10X Sample and Wash Buffer Concentrate: 1 bottle (40 ml) to make 400 ml.

C: Biotinylated Primary Detection Antibody: 1 vial of 100 ul to make 50 ml. (1/2 to be used in assay and the other 1/2 can be saved for subsequent Single-Tope or Selecta-Tope assays.)

D: Horse Radish Peroxidase Conjugated Streptavidin(SAHRP): 1 vial of 1.5 ml to make 30 ml.

E: Chromogenic Solutions : 1 bottle (30 ml) of a stabilized H_2O_2 , 1 vial (1.5 ml) of stabilized TMB chromogen **Caution!**

F: Stop Solution: 1 bottle (30 ml) **Acid Caution!**

Incubation Time and Temperature: An 18 hour EV capture incubation at 4° C is recommended. A 1 hour detection incubation with detection antibodies at room temp is sufficient. Shaking is necessary during all incubations to assure maximum interaction between the EV and the capture antibody. If you alter these times, control them to reproduce your results.

Calibration: Prior to assay, the EV sample should be calibrated using the EVix High Sensitivity Tetraspan Tracking (HSTT) kit to determine presence of EVs in sample. Samples should be sufficiently concentrated to generate HSTT signals above 3.0 AU at 450 nm. A sample volume of 13.0 ml is needed to run the complete 256 well Multi Tope assay.

Assay Protocol: An 18 hour incubation to capture EVs is followed by a 1 hour detection antibody incubation followed by a 20 minute SAHRP incubation. Wells are washed once after detection antibody incubation and three times after SAHRP incubation. The assay should be run in total to reduce inter-plate variation, each plate has control tetraspan signals which can be used to track inter-plate variation.

1. Capture EVs (18 hours): Dispense 100 ul of 1X wash buffer or EV diluent into the odd numbered columns of the three Single-Tope capture plates for negative controls. Plate 1 and 2 each have 96 wells coated with capture antibody. Plate 3 has 64 wells coated with capture antibodies, the last 4 columns (9,10,11,12) wells are uncoated and not used. Dispense 100 ul of your EV sample into wells in the even numbered columns of the Single-Tope plates. Incubate plates for at least 18 hours at 4° C with continuous shaking. Use 1X wash buffer to dilute your sample if necessary and for the negative control wells which do not receive EVs. Refer to map below to fill plates. After 18 hour capture incubation, aspirate the well volume. The well is ready for the next step, the addition of detection antibody.

2. Detection antibody (1 hour): Dilute detection antibody into 1X wash buffer. Remove 50 ul from Ab vial and add to 27 ml of 1X wash buffer. A total of 25.6 ml is needed for the 256 wells. The remaining detection antibody can be used in subsequent assays and should be stored at 4C. Add 100 ul of properly diluted biotinylated detection antibody solution to each empty aspirated well in the assay and incubate plates at room temperature with shaking for 1 hour.

After 1 hour incubation (shaken) , aspirate the well volumes. Add 300 ul of wash buffer to each well and aspirate. The empty well is ready for the next step, addition of 100 ul Streptavidin Horse Radish Peroxidase (SAHRP).

3. SAHRP (20 minutes): Add 100 ul of diluted SAHRP solution to each empty well. SAHRP is diluted 21 fold before use. To make 30 ml of SA HRP add 1.5 ml (vial contents) of concentrate to 30 ml of 1X Wash Buffer. Plates should be shaken at room temp for **20 min**.

4. Wash 3X: After SAHRP incubation, wells receive 3 x 300 ul washes. Well contents are aspirated and 300 ul of 1X Wash Buffer is added. This is done 3 times and final volume is aspirated prior to addition of chromogenic substrate.

5. Chromogen Addition (10 minute): The chromogenic solution is prepared by adding 1.5 ml of part A to 30 ml of part B. **CAUTION: These components are considered hazardous.** Add 100 ul of chromogenic solution to each washed well. The enzymatic reaction is carried out for 10 min and should be timed, to provide equivalent results from assay to assay. Blue color will develop in positive wells.

6. Stop Solution: Add 100 ul of stop solution to all wells after the above 10 minute incubation. Wells will change from blue to yellow upon addition of the stop solution. **CAUTION. Stop solution is 1N Sulfuric Acid and should be handled accordingly.**

7. Read Absorbance: Read the optical absorbance of each well at 450 nm wave length within 10 minutes after addition of the stop reagent.

Data Analysis: The map below shows the capture antibody used for each well in the Single Tope plates. The wells are set up with negative controls (no EV) in the odd numbered columns next to the sample wells in the even numbered columns. The background signal for each capture antibody is subtracted from the sample signal to yield ABS value above background.

	1 , 2	3, 4	5,6	7,8	9,10	11, 12
A	CD9	CD63	CD81	Null	CD9	CD63
B	CD1a	CD6	CD13	CD22	CD30	CD37
C	CD1b	CD7	CD14	CD23	CD31	CD38
D	CD1c	CD8	CD16	CD25	CD32	CD39
E	CD2	CD10	CD18	CD26	CD33	CD40
F	CD3	CD11a	CD19	CD27	bcl-2	CD41
G	CD4	CD11b	CD20	CD28	CD35	MHCII
H	CD5	CD11c	CD21	CD29	MHCI	CD43

MT1 Plate 1

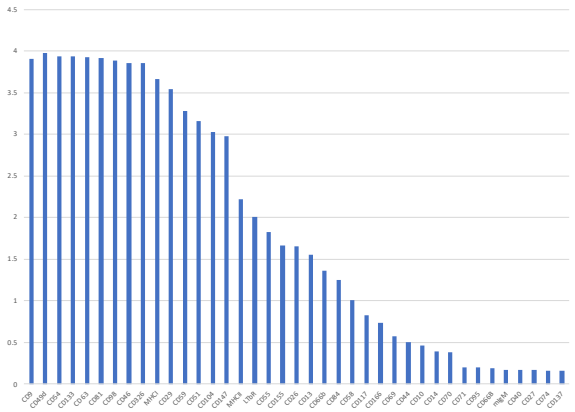
	1 , 2	3, 4	5,6	7,8	9,10	11, 12
A	CD81	Null	CD9	CD63	CD81	Null
B	CD44	CD51	CD62e	CD70	CD84	CD105
C	CD45	CD53	CD62L	CD71	CD86	CD106
D	CD45RO	CD54	CD62P	CD74	TCRCb1	CD117
E	CD46	CD55	CD64	CD79a	CD94	DR3
F	CD48	CD56	CD66b	CD79b	CD95	CD122
G	CD49d	CD58	CD66e	CD80	CD98	CD127
H	CD50	CD59	CD69	CD83	CD104	Perforin

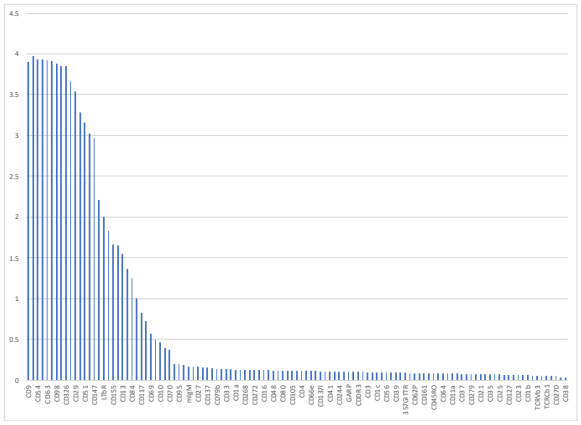
MT2 Plate 2

	1 , 2	3, 4	5,6	7,8
A	CD9	CD63	CD81	Null
B	CD133	CD155	CD270	357GITTR
C	CD134	p53	CD271	CD66B
D	CD137	CD161	CD272	GARP
E	CD137l	CD166	CD273	TCRVb3
F	CD147	CD244	CD274	LTbR
G	CD152	CD252	CD279	TIGIT
H	CD154	CD268	CD326	mIgM

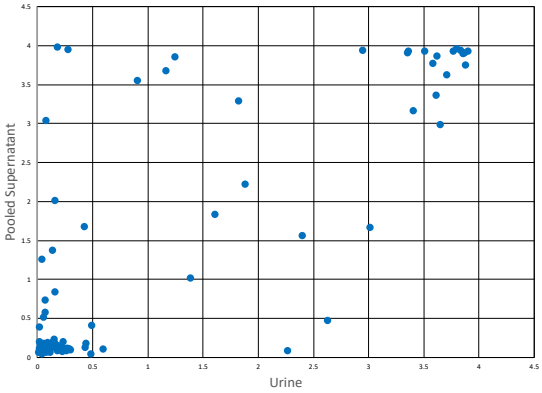
MT3 Plate 3

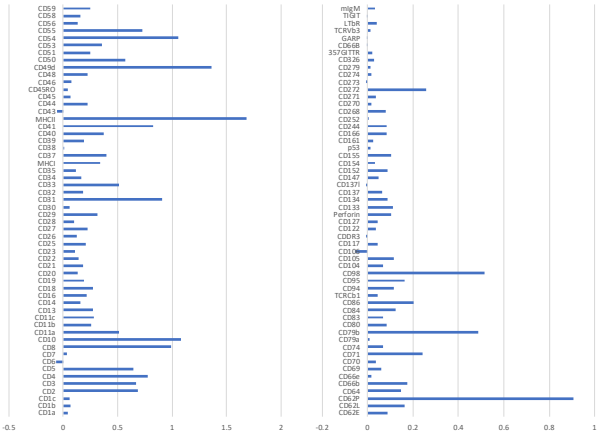
Calibration of sample via EVix HSTT kit prior to assay assures sufficient EVs are present for evaluation. As seen in the values below, CD9, CD63 and CD81 tetraspan capture wells all give near maximum signals. All signals can be ranked and those above a set threshold can be examined and compared to other populations from different samples or sources. Shown below is a sample from pooled culture supernatants with values ranked. The left panel displays only values greater than 0.150. The right panel shows all values with background subtracted. Comparison of different EV populations is done by examining the presence of the antigen positive signals. Different samples and sources yield different profiles.





X Y scatter plots can be created using the Single-Tope assay. Comparison of two different populations is done by finding signals that may be in one population but not the other. These signals will fall along the axis distinct from signals that are present in both populations. As shown in the panel on the right, there are EVs in urine which are positive for a particular antigen but they are not present in the pooled supernatant sample. Other antigens are represented at similar levels in both populations. Additionally, while some antigens may be present in both populations, they may be expressed at different levels.





Single Tope antigen patterns differ significantly depending upon EV source. As shown to the left, 10X concentrated and dia-filtered (300 MWCO) supernatant from a human mixed lymphocyte culture on day 7 yield signals not seen in Urine or serum samples. As would be expected, there are more immune associated antigens detected. The Multi-Tope assay creates a detailed fingerprint of tetraspan associated antigens. The Single-Tope assay also creates a fingerprint with other selected detection antibodies. Comparison of the phenotypes based on detection antibodies can be used to further characterize EV populations. This complex phenotype analysis can be used to confirm similarities in various EV preparations. This is valuable in manufacturing control.

Having identified tetraspan associated antigens in EV populations using the Multi-Tope kit, relevant antigens can now be further characterized. Use the EVix Selecta-Tope kit to characterize the antigen profile of your different positive populations. While positive populations in the Multi-Tope assay, express tetraspans, as they are detected by the mix of anti-tetraspan detection antibodies, the next question is what antigens are expressed on the specific identified populations. The Selecta-Tope kit allows the user to capture with the Multi Tope plates but to screen with the biotinylated detection antibodies of choice. This provides further analysis of EV populations. As example, are EPCAM positive EVs also positive for CD10? Are MHC I positive EVs also MHCII positive?