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Single Extracellular Vesicle Proximity Barcoding Assay (PBA) Kits

16

Version: 1.0

For Research Use Only

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User Guide for KEV0001M16 & KEV0002M16

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01. Introduction

This User Guide describes best practices for procedures to perform the Secretech Proximity Barcoding Assay (PBA) applied to single extracellular vesicles (EV) using the Secretech kits: KEV0001M16 (for Human derived EVs) and KEV0002M16 (for Mouse derived EVs) kits.

The assay renders as output a PBA library ready for NGS sequencing. From the sequencing data, single extracellular vesicles proteomic data can be decoded and analyzed. Secretech provides its Secretech EVisualizer® software for initial analysis of data and the visualization of the single EV subpopulations.

The protocol is structured over three days:

- **Day 1:** EV capture plate preparation
- **Day 2:** sample preparation and PBA
- **Day 3:** library preparation and quality control

02. Equipment and materials not provided

The following check list provides you with a reference to equipment you need to have available and reagents and consumables that you need in PBA, and which are not provided with the kits: KEV0001M16 and KEV0002M16.

level:

* must have;
\# highly recommended;

Instruments

Check	Items	Level	Description
☐	2–8 °C fridge	*	Reagent kit storage
☐	-20 °C freezer	*	Reagent kit storage
☐	-80 °C freezer	#	Sample storage
☐	Biosafety level 2 cabinet	#	Sample handling
☐	4 °C Centrifuge up to 10,000 × g for 1.5 mL tubes	#	Sample treatment
☐	Bench top centrifuge for 1.5 mL tubes	*	Spinning down the reagent in the tubes
☐	Centrifuge for plates	*	Spinning down the reagent in the plates
☐	Bench top vortex with adaptor for plate	*	Mixing of reagent or samples
☐	Vacuum pump connected to a bottle for waste liquid; with an 8-channel tip adaptor in the tubing system	*	Removal of liquid from 96-well plates
☐	Pipet set (1000 µL; 100 or 200 µL ; 10 or 20 µL)	*	Reagent handling
☐	8-channel pipet set (10 µL, 100 µL)	*	Reagent handling
☐	8-channel pipet (300 µL)	#	Reagent handling
☐	Plate incubator (37–95 °C) or thermo-shaker	*	Incubation of plates
☐	Real time PCR instrument	*	Incubation and PCR
☐	Library size QC devices, e.g. bioanalyzer or DNA gel electrophoresis system	#	PBA library QC: size of 220 bp
☐	Library quantification devices, e.g. Qubit with dsDNA kit or other techniques	#	PBA library QC: library concentration
☐	Sequencing instrument or sequencing service	*	PBA library sequencing

Consumables

Check	Items	Level	Description
☐	PCR plates adapted to your PCR instrument (at least 3 plates)	*	Collection of treated samples and PCR
☐	Large reagent tube or bottle (50–500 mL)	*	Washing buffer dilution
☐	8-well strips or cluster tubes (at least 10 strips)	*	Reagent dispensing with 8-channel pipets
☐	Reagent reservoir	*	Buffer dispensing with 8-channel pipets
☐	Pipet tips (PCR grade) adapted to your pipets	*	Liquid handling
☐	Sealing bags	#	Storage of PCR plates

Reagents

Check	Items	Level	Description
☐	Phosphate buffered saline (PBS), 500 mL	*	Sample dilution
☐	Ultrapure water (suitable for PCR), 300 mL	*	Buffer dilution
☐	Kit for PCR product purification	*	Purification of PBA library

03. PBA Kit Components

Amounts of reagents and consumables provided in KEV0001M16 and KEV0002M16 are sufficient for running up to 16 samples in parallel on a 96-well capture plate. The two parts of kit need to be stored according to the tables below, upon arrival.

Box A – Store at 4 °C

Code	Items	Description	Size
R2	Coating buffer	Reaction buffer for EV capture plate coating	0.9 mL
R3	20× Washing Buffer	For washing steps	2 mL
C1	EV Capture Plate	For capture of the EVs	1 plate
C2	Incubation Films	For incubation steps	5 pcs
C3	Aluminum Films	For deep-freeze storage of plates	3 pcs
C4	PCR Films	For plate sealing during PCR	3 pcs

Box B – Store at -20 °C

Code	Items	Description	Size
R1	100× EV Capture Reagent	Reagent for EV capture plate coating	10 µL
R4	PBA Probes	For protein detection	16 µL
R5	PBA Buffer	Reaction buffer for protein detection with PBA probes	0.5 mL
R6	PBA Template	Template for single EV barcoding	0.5 mL
R7	Barcoding Solution	For the extension reaction in EV barcoding	0.5 mL
R8	Releasing Solution	For protein degradation and DNA releasing	0.5 mL
R9	1st PCR Solution	Reagent for the first PCR reaction	0.25 mL
R10	2nd PCR Plate	For sample indexing reaction	1 plate
R11	3rd PCR Solution	Reagent for the final PCR steps	0.25 mL

04. PBA Procedure

Day 1 (1 hour hand-on time): preparation of EV capture plate

A. Preparation of EV capture plate

1. Prepare 0.9 mL EV capture plate coating solution by mixing 9 µL 100 × EV Capture Reagent (R1) with 891 µL Coating buffer (R2) in a clean tube and vortex to mix the solution.
2. Dispense coating solution (R1+R2) 50 µL/well into EV Capture Plate (C1).
3. Seal the plate (C1) with incubation film (C2) and leave the plate overnight in the refrigerator (2–8 °C).

NOTE: The bottom of the wells should be completely covered by the solution and be free of bubbles.

Day 2 (6–8 hours hand-on time): sample preparation and PBA

B. Sample preparation

4. Thaw the samples (stored at -80 °C) under room temperature (20–25 °C). Vortex to mix the samples well. Centrifugation of samples (2000 × g for 10 min) is recommended for removal of aggregates.

NOTE: Samples should be prepared in accordance to guidelines of MISEV2023 (PMID: 38326288) and literatures. The centrifugation at 2000 × g is only for pre-PBA treatment of qualified EV samples, but not for treatment of original samples like whole blood etc.

5. Transfer the supernatant of each sample to a new tube for sample dilution if needed.

NOTE: Sample dilution should be performed to avoid over-loading of EVs in each PBA test. Secretech provides the experience-based recommendation on the dilution factors depending on the types of samples. However, due to batch difference, the following sample dilution method is not gold standard. Optimize the sample loading amount if necessary.

NOTE: about the batch effect: A) Concentration of EVs in a certain sample type varies depending on the method of isolation and processing and biological condition at the moment of sample collection. B) Concentration measurements vary and are dependent on the method and parameters used.

Sample type		Dillution	Loading volume	Sample loading rationale
Purified EVs in PBS	Concentration measured with NTA	Diluted each sample to 1.0×10^{11} particle/mL with PBS	30 µL	EVs with the same measured particle number
	Concentration measured with flow cytometer	Diluted each sample to 1.0×10^{10} particle/mL with PBS	30 µL	
	Concentration measured for 3 samples per group	Dilution factor is determined from average conctraiton and applied to every sample	30 µL	EVs purified from the same amount of original samples
Body fluids (BF)	Plasma or Serum	5 µL BF + 495 µL PBS	30 µL	EVs from the same volume of original BF
	CSF or urine	20 µL BF + 40 µL PBS	30 µL	
	Other body fluids	10 µL BF + 50 µL PBS	30 µL	
Low concentration EV samples (challenging)	Cell culture medium or irrigation solutions	No dilution	30 µL loading for 1–3 times	EVs from the same volume of original samples
Purified EV without concentration measurement (challenging)	Due to the uncertainty of EV recovery rate of various purification method. A general sample loading guide cannot be provided. We suggest to perform a preliminary PBA test with 8 sample PBA kit. By loading sample with a series of dilution factor, the proper loading condition could be determined from the results.			EVs from the same amount of original samples

6. Store the original remaining samples back to -80 °C.
7. Diluted samples can be transferred into a 96-well plate and kept at ambient temperature till use in the next steps.
8. If needed, positive and negative controls could be added to each batch of PBA test. Prepare your Positive control samples in the same way. PBS (1×) could be used as Negative control.

C. Wash the EV capture plate

9. Prepare the Washing buffer working solution (1×) by diluting 20× washing buffer (R3) with DNAse/RNAse free ultrapure water (dilution 1:19). Vortex to mix the washing buffer thoroughly.

NOTE: 1920 µL/well of washing buffer (1×) are needed for the whole PBA process. (a 10 % extra volume considering pipetting errors is already accounted for in this value).

10. Bring from overnight storage the coated EV Capture plate (C1) and proceed to wash the plate with the following 3-step washing procedure using the Washing buffer working solution (1×).

3-step (80–100–120) washing procedure:

i. Remove the solutions in each well with an aspirator pump or multichannel pipette.
ii. Add 80 µL 1× washing buffer to each well.
iii. Remove the washing buffer and add 100 µL 1× washing buffer to each well.
iv. Remove the washing buffer and add 120 µL 1× washing buffer to each well.
v. Make sure the working solution for next step is ready to be added to the well immediately after final washing step.
vi. Remove the washing buffer and put the plate upside down on several layers of clean tissue paper.
vii. Pat the plate carefully until no excess liquid left.

D. EV capture

11. Load 30 µL per well of the diluted samples and controls to the washed EV capture plate (C1). Avoid bubbles and centrifuge briefly to remove them if necessary.
12. Seal the EV capture plate (C1) with incubation film (C2) and incubate the plate at room temperature for 1 hour.

E. Antibody binding

13. During the above 1-hour incubation prepare the PBA Probes Solution by adding all the PBA Probes (R4) to PBA Buffer (R5). Mix the solution thoroughly by pipetting up and down gently. DO NOT Vortex!
14. After the 1-hour incubation, wash the plate (C1) from step 12) with the 3-step washing procedure.
15. Add 30 µL PBA Probes Solution from step 13) to each well in EV capture plate (C1). Avoid bubbles.
16. Seal the plate C1 with incubation film (C2) and incubate the plate at room temperature for 2 hours.

F. PBA template binding

17. During the above 2-hour incubation time, place the PBA template solution (R6) at room temperature to thaw. DO NOT Shake or Vortex! Mix the solution thoroughly by pipetting up and down gently.
18. Turn on the heating on a thermoblock for plates with the temperature setting to 37 °C. (Needed for step 21).
19. After the 2-hour incubation time from step 16), wash the plate with a 5-step (80–100–120–120–120) washing procedure.

    5-step (80–100–120–120–120) washing procedure:

    Based on the 3-step wash, add two more steps of 120 µL washes.

20. Add 30 µL/well of PBA template (R6) to the washed EV capture plate (C1).
21. Seal the EV capture plate (C1) with incubation film (C2) and incubate the plate at 37 °C for 15 min on the pre-heated thermoblock.

G. EV Barcoding

22. During the above incubation time, place the Barcoding solution (R7) at room temperature to thaw. DO NOT Shake or Vortex! Mix the solution thoroughly by pipetting up and down gently.
23. Wash the plate C1 from step 21) with the 3-step washing procedure.
24. Add 30 µL/well of the Barcoding Solution (R7) to the washed EV capture plate (C1). Avoid bubbles.
25. Seal the EV capture plate (C1) with incubation film (C2) and incubate the plate at room temperature for 20 min.
26. Set the thermoblock for plates to 50 °C and leave it on heating. (Needed for Step 31).

H. DNA releasing

27. During the above incubation time, place the Releasing solution (R8) at room temperature to thaw. Mix the solution thoroughly by pipetting up and down.
28. After the 20-min incubation, wash the plate C1 from step 25) with the 3-step washing procedure.
29. Add 30 µL Releasing solution (R8) to each well of the EV capture plate (C1). Avoid bubbles.
30. Seal the EV capture plate (C1) with incubation film (C2) and incubate the plate at 50 °C for 15 min.
31. During the incubation time, label a new 96-well PCR plate (compatible with your Real time thermal cycler) as the 1st PCR Plate.
32. After this incubation in step 30), gently shake or vortex the plate (C1) at low speed. Spin down the content in the plate briefly.
33. Mix the solution in each well of plate C1 by pipetting and transfer the solutions to corresponding well in the 1st PCR plate. Keep the same sample positions in plates.
34. Seal the 1st PCR plate with a new film (C2) and run it at 90 °C for 10 min in a thermal cycler with the lid heated to 105 °C.
35. The EV capture plate (C1) could be discarded after sealing.

I. The 1st PCR reaction

36. During the above incubation time, place the 1st PCR Solution (R9) at room temperature to thaw. Mix the solution thoroughly by pipetting up and down.
37. After the incubation step 34), cool the 1st PCR plate down to room temperature. Spin down the plate briefly.
38. Add 15 µL/well of 1st PCR Solution (R9) to each well in the 1st PCR plate from step 37).
39. Seal the 1st PCR plate with a PCR film (C4) and run the first PCR program:

37 °C — 15 min
95 °C — 2 min
95 °C — 15 s
58 °C — 30 s # — **25 cycles**
72 °C — 30 s
10 °C — 10 min

is for fluorescence measurement of SYBR Green I.

40. Remove the 1st PCR plate from the thermal cycler when the program is finished, briefly spin down the plate.
41. Store the 1st PCR plate at -20 °C if the rest of the protocol will be performed in another day.

NOTE: The CT values of samples and controls are suggested to be measured and compared. It is an indirect check step to make sure the reliability and stability of samples. The CT value could be used as criteria for determination of sequencing reads needed in NGS. Refer to the PBA library sequencing guide for more detail.

Day 3 (4–6 hours hand-on time) library preparation and quality control

J. The 2nd PCR reaction

NOTE: If you run PBA tests very often, use a separate post-PCR room for the rest of this protocol to avoid contamination of PCR product in the future PBA tests.

42. Thaw the 2nd PCR Plate (R10) under room temperature.

43. Thaw the 1st PCR plate from step 41) under room temperature. Vortex the plate gently to mix the solution thoroughly and spin down the solution in the 1st PCR plate.
44. Transfer 1.5 µL PCR product from each well of the 1st PCR plate to corresponding wells in the 2nd PCR plate (R10).

Important: Keep tracking sample distribution on the new plate as well, as this step adds the specific sample sequencing indexes.

45. Seal the 2nd PCR plate (R10) with a new PCR film (C4). Vortex the plate gently to mix the solution thoroughly and spin down the solution in the 2nd PCR plate (R10).
46. Seal the 1st PCR plate with a new aluminum film (C3) and save it inside a sealed plastic bag and store at -20 °C.
47. Run the 2nd PCR plate with following this program:

95 °C — 2 min
95 °C — 15 s
60 °C — 1 min } **2 cycles**
72 °C — 1 min
72 °C — 3 min

48. Remove the 2nd PCR plate from the thermal cycler when the program is finished and briefly spin down the plate.

K. The 3rd PCR reaction

49. Thaw the 3rd PCR solution (R11) under room temperature. Mix well by gently pipetting.
50. Dispense 13.5 µL of 3rd PCR Solution (R11) to each well of a new 96 well PCR plate, which could be marked as the 3rd PCR plate.
51. Transfer 1.5 µL of the products from the 2nd PCR plate to the corresponding wells in the 3rd PCR plate. Keep the same sample distribution in wells as 2nd PCR plate.
52. Seal the 3rd PCR plate with a new PCR film (C4). Mix well and spin the solution down.

53. Seal the 2nd PCR plate with a new aluminum film (C3) and put into a sealing plastic bag and store at -20 °C.

54. Run the 3rd PCR plate using the following program:

95 °C — 2 min
95 °C — 15 s
60 °C — 30 s # } **30 cycles**
72 °C — 30 s
10 °C — 90 min

is for fluorescence measurement of SYBR Green I.

NOTE: The PCR amplification curve of each sample should have reached its plateau phase at cycle 30.

55. Remove the 3rd PCR plate from the thermal cycler when the program is finished and briefly spin down the plate.

L. Library pooling

56. Pool together 3 µL of the 3rd PCR products from each of samples to be sequenced.
57. Seal the 3rd PCR plate with a new aluminum film (C3) and put into a sealing plastic bag and store at -20 °C.

M. PCR Product Purification

58. Purify the pooled samples with a purification kit suitable for DNA fragments of 200–250 bp, while the PBA library was expected to be about 220 bp.

NOTE: kits that can be used: TaKaRa MiniBEST DNA Fragment Purification Kit Ver.4.0; MagMAX™ Pure Bind Beads (Thermo-Applied Biosystems); GeneJET PCR Purification Kit (Thermo); QIAquick PCR Purification kit (Qiagen) or similar from other vendors for purification of PCR products for NGS/Sequencing.

59. Collect the purified PCR product into a new 1.5/2 mL tube and label the tube as PBA_Library.

NOTE: All the PCR plate can be stored in freezer (-20 °C) and then discarded after you finish the PBA project successfully.

N. Library QC

60. **DNA fragment size:** The fragment size for the PBA library is about 220 bp. Depending on the method you use for DNA fragment analysis, a peak of the library is expected in the range of 210–240 bp.
61. **Library concentration:** Measure the DNA concentration of the PBA library using Qubit with dsDNA HS Assay Kit or similar method. The concentration is needed for library loading in NGS. Dilute or prepare the library for sequencing or submit to a Sequencing facility following their guidelines.

O. NGS Library Sequencing

62. Single end sequencing of 75 cycles + dual index sequencing of 8 cycles are needed when choosing NGS platform and reagent kits. Please refer to the document of PBA library NGS setup for more information.

NOTE: The PBA library is compatible with sequencing kits with larger cycling setup, e.g. SE100, PE100, PE150 and so on. Though the extra bases after SE75 will not contribute to the data analysis.

P. Data Analysis

63. Please refer to the document of PBA Data Processing. Secretech provides PBA bioinformatic tools for sequencing data decoding and further single EV proteomic analysis.

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Email: info@secretech.com
Web: www.secretech.com