Supplementary Information for

Sequencing-Free, Joint Single-EV Profiling of DNA and Protein Cargos Enables Accurate Cancer Detection at Early Stages

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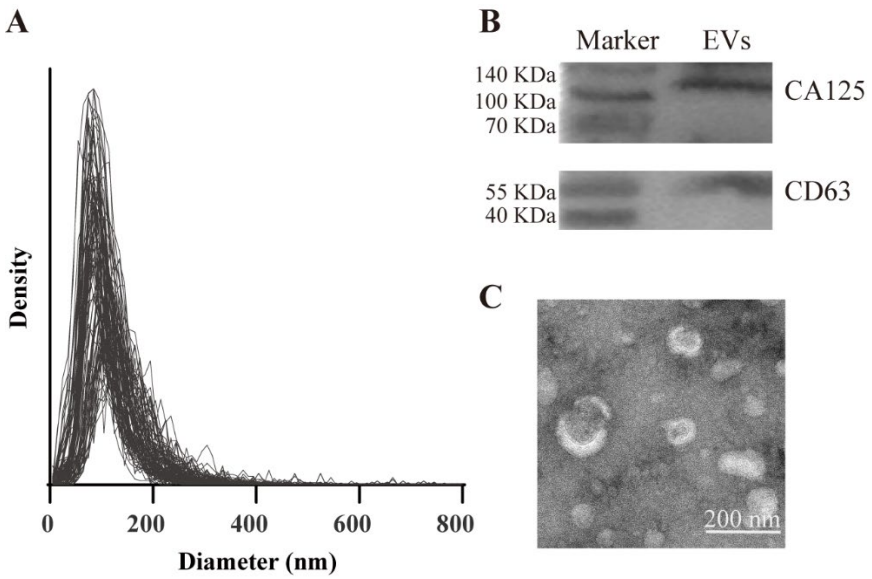

Fig. S1 Characterization of EV samples. (A) The particle size distributions of EVs from each plasma sample determined by nanoparticle tracking analysis. (B) The presence of CD63 and CA125 in EVs was confirmed by Western blot using anti-CD63 (25682-1-AP, Proteintech group) and anti-CA125 (14497-1-AP, ab134093). (C) TEM showed the typical cup-like morphology and membrane structure of EVs.

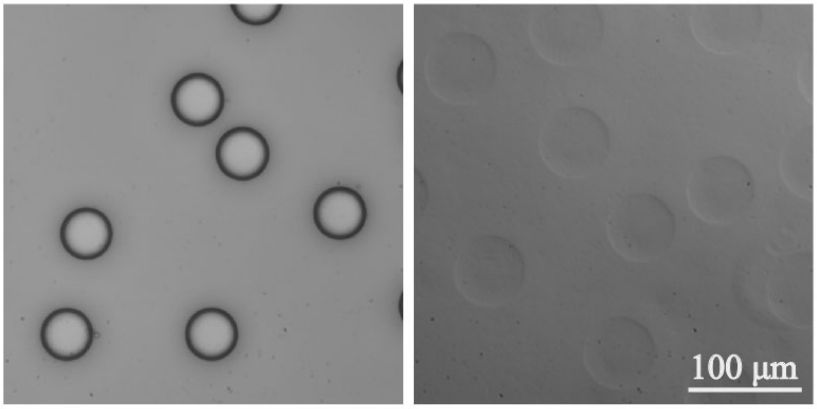

Fig. S2 Hydrogel droplets in a microfluidic chamber. Replacing the mineral oil with an aqueous solution (PBS) leads to swelling of the hydrogel droplets.

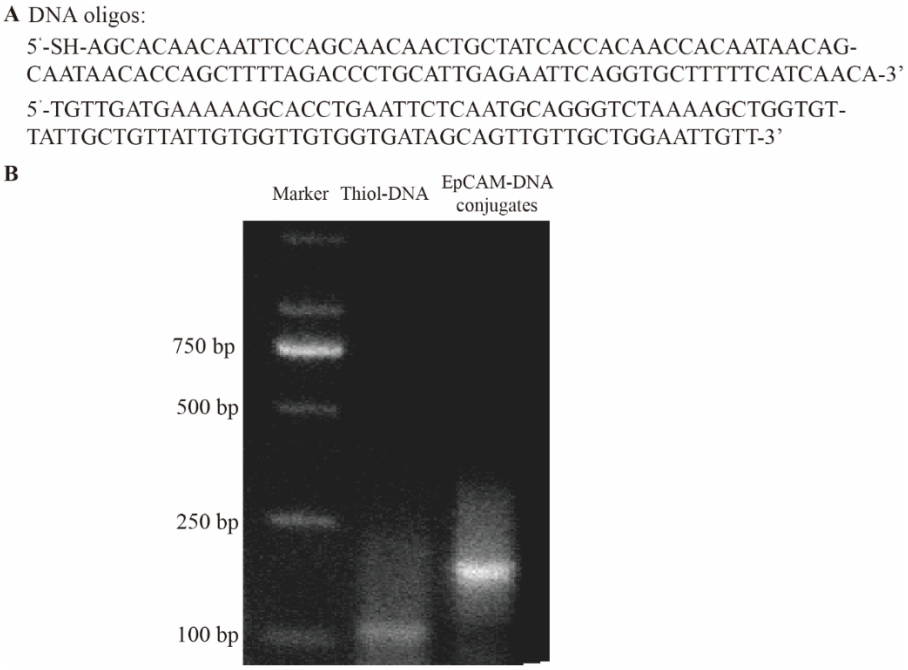

Fig. S3 EpCAM-DNA conjugates. (A) The randomly generated sequence of 105-nt DNA oligonucleotides, which was modified with a sulfhydryl group at the 5'-end, and its 100-nt complementary sequence. To prepare double-stranded DNA, 2 nmol of each the DNA oligonucleotides (Tsingke Bio) were dissolved and mixed in 20 μL PBS (pH 7.4), and heated to 95°C to denature the strands, followed by gradual cooling to allow annealing. (B) Gel electrophoresis analysis of the double-stranded DNA (Lane 2) and EpCAM-DNA conjugates (Lane 3). The band in Lane 3 is noticeably delayed compared to the band in Lane 2, indicating the forming of protein-DNA conjugates.

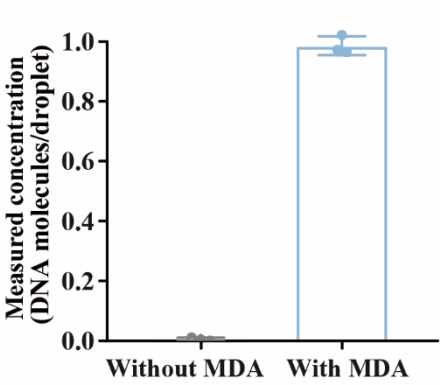

Fig. S4 MDA ensures single-molecular sensitivity for DNA detection. A DNA ladder was spiked in PEG hydrogel at a concentration of 1.0 molecule per droplet volume. Data presented as means ± SD (n = 3).

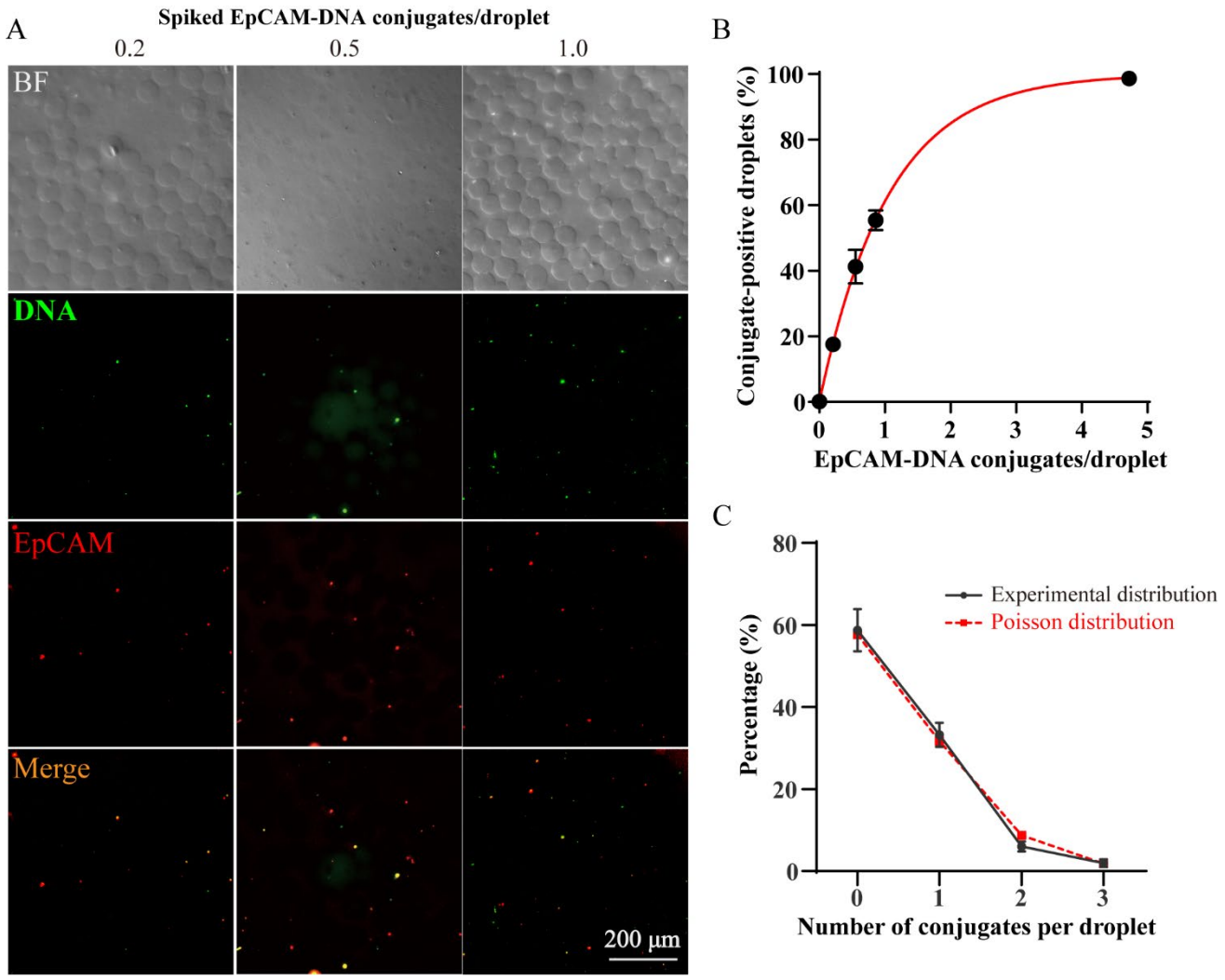

Fig. S5. EpCAM-DNA conjugate detection with nearly single-molecular sensitivity. (A) Typical images showing aligned fluorescent signals of EpCAM (red) and DNA (green) in detecting EpCAM-DNA conjugates. (B) Theoretical and measured proportions of conjugate^{+} droplets at different conjugate concentrations. (C) The distribution of EpCAM-DNA conjugates at a concentration of 0.55 conjugate/droplet among hydrogel droplets obeys the Poisson distribution, which suggests minimal signal overlap.

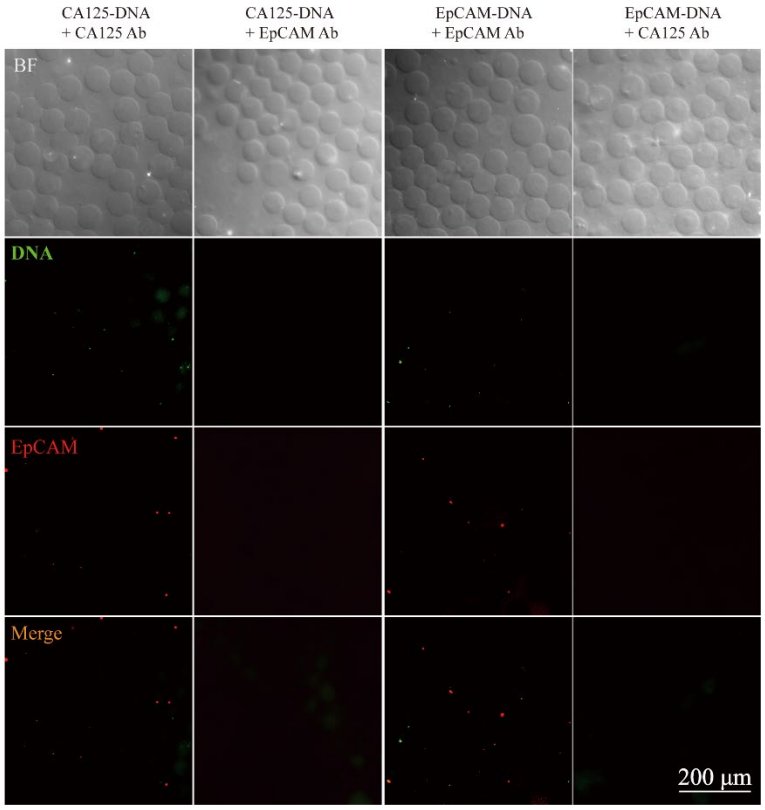

Fig. S6 Target-specificity of the assay evaluated with different protein-DNA conjugates and fluorescent antibodies.

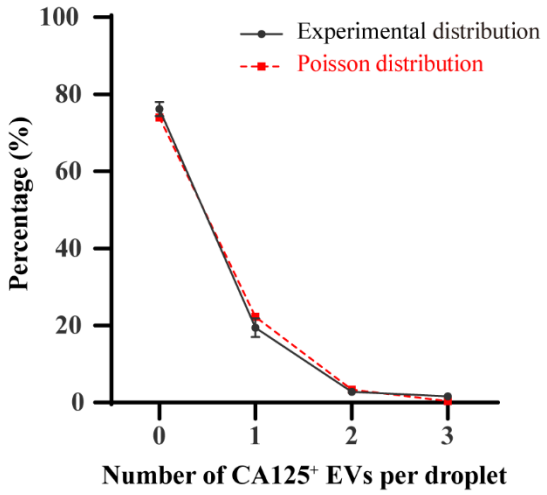

Fig. S7 The distribution of EVs among hydrogel droplets obeys the Poisson distribution, indicating minimal signal overlap and a low false-positive rate of the single-EV assay. For plasma sample #88, EVs were dispensed into droplets at a concentration of 5.0 particles/droplet volume, and the P($CA125^+$ EV) was determined to be 6.02%, resulting in a final concentration of 0.301 $CA125^+$ EV/droplet. Experimental and theoretical distributions were plotted for comparison.

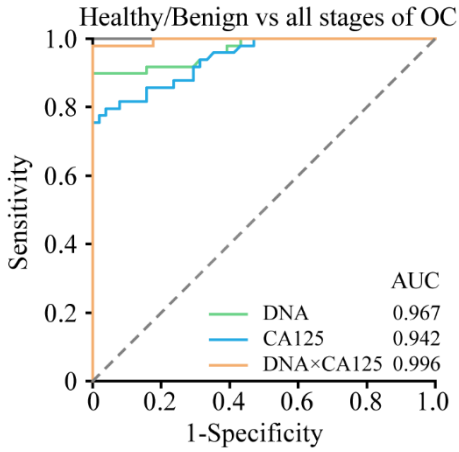

Fig. S8 ROC curves for P(DNA$^+$ EV), P(CA125$^+$ EV), and P(CA125$^+$DNA$^+$ EV) to differentiate all stages of OC (n = 49) from healthy controls and benign diseases (n = 51).

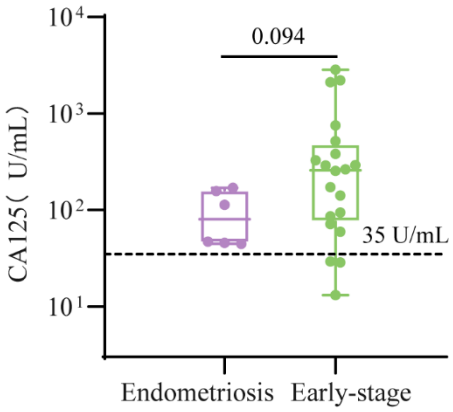

Fig. S9 Blood CA125 level of patients with endometriosis (n = 6) and early-stage OC (n = 21).

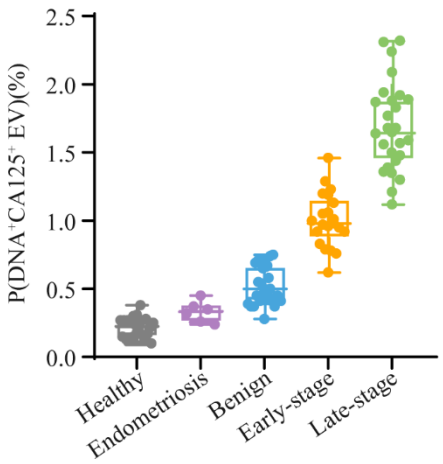

Fig. S10 P(CA125$^+$DNA$^+$ EV) determined for EV samples derived from healthy (n = 22), endometriosis (n = 6), benign diseases (n = 29), early-stage OC (n = 21), and late-stage OC (n = 28) groups.

Table S1 The blood CA125 level and single-EV analysis data determined for each clinical sample in the study.

Group	OC stage	Sample No.	Blood CA125 level (U/mL)	EV yield (10^{10}) from 200 μL of plasma	Percentage of positive EVs (%), n ≥ 3					
					DNA^+ EV		$CA125^+$ EV		DNA^+CA125^+ EV	
					Mean	SD	Mean	SD	Mean	SD
Healthy (N=22)	NA	1	Not determined	3.30	0.86	0.04	1.68	0.05	0.30	0.01
		2		0.97	0.82	0.04	1.32	0.07	0.13	0.01
		3		1.20	0.48	0.02	1.45	0.07	0.25	0.02
		4		0.95	0.71	0.04	1.15	0.05	0.27	0.02
		5		0.34	0.58	0.03	0.82	0.04	0.31	0.01
		6		0.36	0.56	0.03	1.21	0.05	0.21	0.02
		7		0.76	0.66	0.03	0.97	0.05	0.13	0.02
		8		0.90	0.90	0.04	0.91	0.02	0.13	0.01
		9		0.54	0.93	0.02	1.13	0.04	0.27	0.01
		10		3.65	0.60	0.06	0.96	0.04	0.15	0.01
		11		3.35	0.84	0.04	1.32	0.07	0.27	0.02
		12		5.25	0.67	0.04	1.22	0.05	0.27	0.03
		13		2.70	0.84	0.02	1.38	0.02	0.17	0.03
		14		1.40	0.52	0.05	1.02	0.05	0.38	0.03
		15		1.50	0.76	0.04	1.06	0.03	0.28	0.02
		16		8.20	0.66	0.02	0.80	0.07	0.18	0.02
		17		4.20	0.60	0.04	1.22	0.04	0.15	0.01
		18		1.15	0.70	0.03	1.11	0.03	0.24	0.03
		19		2.90	0.58	0.05	1.17	0.04	0.21	0.02
		20		11.00	0.53	0.04	1.10	0.07	0.10	0.02
		21		2.30	0.70	0.06	1.35	0.05	0.27	0.03

		22		1.10	0.70	0.06	1.29	0.06	0.17	0.01
		23	5.44	2.10	1.07	0.05	1.07	0.05	0.44	0.01
		24	200	5.30	0.88	0.04	2.47	0.08	0.45	0.02
		25	12.9	0.12	1.54	0.04	1.43	0.05	0.74	0.03
		26	20.3	0.70	1.43	0.02	1.74	0.04	0.37	0.02
		27	575	1.85	1.15	0.02	2.82	0.05	0.65	0.03
		28	87.3	0.37	1.21	0.04	2.07	0.11	0.69	0.03
		29	28.4	4.55	1.30	0.02	1.89	0.09	0.55	0.03
		30	8.74	5.70	1.32	0.04	1.08	0.04	0.71	0.02
		31	11.3	3.65	0.69	0.03	1.29	0.05	0.37	0.02
		32	10.3	3.70	1.14	0.05	1.70	0.06	0.67	0.01
		33	25.3	0.29	0.50	0.03	1.96	0.07	0.28	0.01
Benign		34	9.04	3.50	1.37	0.02	1.96	0.04	0.41	0.04
ovarian		35	11.3	0.13	0.79	0.06	1.38	0.05	0.41	0.04
	NA									
diseases		36	23.2	3.60	1.07	0.06	2.33	0.09	0.45	0.04
(N=29)		37	5.4	4.65	1.22	0.09	1.65	0.05	0.54	0.03
		38	8.56	2.20	0.86	0.06	1.71	0.06	0.50	0.04
		39	51.9	2.25	1.40	0.03	2.03	0.05	0.66	0.04
		40	10.2	2.30	1.29	0.06	2.33	0.07	0.37	0.02
		41	27.3	4.30	0.88	0.08	2.44	0.04	0.41	0.04
		42	6.76	2.50	1.30	0.02	1.64	0.07	0.39	0.03
		43	13.64	1.00	1.33	0.07	2.05	0.07	0.69	0.05
		44	10.3	1.63	1.13	0.08	1.52	0.09	0.43	0.02
		45	13.3	3.00	1.10	0.05	2.32	0.06	0.58	0.04
		46	4.82	2.10	1.35	0.04	1.78	0.06	0.71	0.03
		47	9.85	2.20	1.16	0.09	1.96	0.05	0.47	0.03
		48	46.73	3.10	1.37	0.07	2.20	0.09	0.44	0.03

		49	8.03	3.70	0.83	0.04	1.54	0.08	0.50	0.01
		50	80.1	1.80	1.40	0.08	2.34	0.04	0.75	0.02
		51	33.3	2.95	1.43	0.05	2.52	0.09	0.58	0.04
	I	52	71.8	1.05	0.94	0.05	1.99	0.08	0.62	0.03
	I	53	29.3	1.37	1.56	0.06	1.53	0.06	0.95	0.04
	I	54	28.7	5.50	1.14	0.07	1.65	0.04	0.76	0.03
	I	55	59.7	3.10	1.16	0.07	1.90	0.06	0.78	0.04
	I	56	383	0.72	2.12	0.05	3.05	0.06	0.92	0.04
	I	57	2856	0.62	1.97	0.07	2.37	0.09	0.96	0.04
	I	58	86.3	3.00	1.09	0.09	1.81	0.06	0.79	0.05
	I	59	265	0.22	2.40	0.06	1.74	0.04	0.83	0.06
	I	60	13.2	2.30	1.55	0.07	3.22	0.10	1.29	0.06
Early-stage	II	61	289	2.85	1.58	0.08	2.29	0.09	1.06	0.06
ovarian cancer	II	62	254	4.28	1.61	0.08	2.31	0.06	0.92	0.06
(N=21)	II	63	327	1.10	2.05	0.08	3.13	0.07	1.20	0.04
	II	64	2117	1.70	2.67	0.10	3.73	0.06	1.46	0.07
	II	65	755	4.20	2.01	0.07	3.38	0.06	1.23	0.05
	II	66	173	5.20	1.88	0.09	1.95	0.09	1.05	0.03
	II	67	141	2.10	1.92	0.07	2.97	0.09	1.19	0.05
	II	68	293	8.50	1.34	0.04	3.56	0.12	1.00	0.06
	II	69	520	2.10	2.08	0.04	3.34	0.05	1.13	0.05
	II	70	2211.99	1.80	2.60	0.08	3.12	0.11	1.01	0.06
	II	71	/	6.95	1.64	0.05	2.51	0.11	0.97	0.05
	II	72	94.4	2.30	1.66	0.07	2.54	0.05	0.98	0.09
Late-stage	III	73	721	0.52	2.10	0.08	6.75	0.08	1.39	0.06
ovarian cancer	III	74	1145	2.05	2.10	0.08	6.47	0.09	1.35	0.04
(N=28)	III	75	12.9	4.60	2.41	0.06	3.93	0.09	1.56	0.05

	III	76	627	5.08	1.75	0.07	5.44	0.11	1.12	0.05
	III	77	751	2.00	2.50	0.09	5.82	0.12	1.48	0.06
	III	78	72.4	1.95	2.62	0.06	5.07	0.09	1.65	0.08
	III	79	2780	11.00	1.92	0.10	6.89	0.13	1.30	0.06
	III	80	3024	6.20	3.36	0.12	7.08	0.17	1.92	0.07
	III	81	66.8	7.95	2.41	0.11	3.99	0.18	1.57	0.07
	III	82	3109	1.30	3.30	0.15	7.00	0.33	2.09	0.06
	III	83	606	5.80	3.98	0.13	5.56	0.14	1.77	0.07
	III	84	11749	1.22	4.54	0.15	7.73	0.13	2.24	0.05
	III	85	49	0.61	2.28	0.07	4.84	0.12	1.50	0.07
	III	86	57	3.20	2.39	0.06	4.50	0.08	1.87	0.06
	III	87	953	0.20	3.21	0.08	5.12	0.08	1.44	0.07
	III	88	1224	3.45	3.44	0.06	6.02	0.16	1.83	0.02
	III	89	328	2.60	2.02	0.06	4.63	0.07	1.21	0.04
	III	90	172	10.00	2.05	0.04	5.66	0.08	1.36	0.06
	III	91	574	10.23	2.65	0.07	5.87	0.09	1.59	0.05
	III	92	2408	0.63	2.99	0.09	6.58	0.16	2.31	0.05
	III	93	10237	1.05	3.25	0.09	7.35	0.12	1.94	0.03
	III	94	214	0.48	1.81	0.08	4.45	0.08	1.68	0.07
	III	95	1000	4.50	2.55	0.07	5.67	0.10	1.64	0.14
	III	96	1818	0.89	2.63	0.06	6.35	0.12	1.58	0.06
	IV	97	2512	0.56	2.82	0.12	6.30	0.14	1.89	0.09
	IV	98	1897.87	0.94	3.92	0.14	5.37	0.13	2.32	0.10
	IV	99	1830	0.99	2.99	0.15	5.84	0.19	1.88	0.09
	IV	100	1646	7.20	3.12	0.07	6.25	0.10	1.88	0.07
Endometriosis (N=6)	NA	101	170	2.20	0.80	0.04	2.04	0.09	0.35	0.02

102	158	2.85	0.68	0.03	1.62	0.06	0.24	0.01
103	44.8	2.63	0.76	0.03	0.80	0.04	0.32	0.02
104	45.8	1.80	0.73	0.04	1.58	0.07	0.37	0.02
105	47.2	2.10	1.01	0.05	1.02	0.05	0.26	0.02
106	113	4.35	0.73	0.04	1.65	0.04	0.45	0.03

Table S2 Details for ROC curve analysis.

Figure	Groups in comparison	Marker	AUC	95% CI	Optimal cut-off point	Sensitivity	95% CI	Specificity	95% CI
2C	Healthy vs Benign	DNA^+ EV	0.922	0.844 to 1.000	1.000	0.759	0.579 to 0.878	1.000	0.851 to 1.000
	Healthy vs Early-stage		1.000	1.000 to 1.000	0.935	1.000	0.845 to 1.000	1.000	0.851 to 1.000
	Benign vs Early-stage		0.864	0.748 to 0.980	1.545	0.762	0.549 to 0.894	1.000	0.883 to 1.000
	Early-stage vs Late-stage		0.890	0.800 to 0.979	2.090	0.821	0.644 to 0.921	0.810	0.600 to 0.923
S8	Healthy/ Benign vs All stages of OC	DNA^+ EV	0.967	0.935 to 0.988	1.545	0.898	0.782 to 0.956	1.000	0.930 to 1.000
		$CA125^+$ EV	0.942	0.901 to 0.983	2.490	0.796	0.664 to 0.885	0.961	0.868 to 0.993
		$CA25^+DNA^+$ EV	0.996	0.989 to 1.000	0.755	0.980	0.893 to 0.999	1.000	0.930 to 1.000
4G	Benign vs Early-stage	Blood CA125	0.910	0.829 to 0.992	55.800	0.850	0.640 to 0.948	0.862	0.694 to 0.945
		DNA^+ EV	0.864	0.748 to 0.980	1.545	0.762	0.549 to 0.894	1.000	0.883 to 1.000
		$CA125^+$ EV	0.767	0.631 to 0.903	2.490	0.524	0.324 to 0.717	0.931	0.780 to 0.988
		$CA25^+DNA^+$ EV	0.985	0.955 to 1.000	0.755	0.952	0.773 to 0.998	1.000	0.883 to 1.000

		LDA-weighted sum	0.985	0.955 to 1.000	0.286	0.952	0.773 to 0.998	1.000	0.883 to 0.998
5B	Endometriosis vs Early-stage	DNA^+ EV	0.992	0.967 to 1.000	1.050	0.952	0.773 to 0.998	1.000	0.610 to 1.000
		$CA125^+$ EV	0.917	0.798 to 1.000	1.695	0.905	0.711 to 0.983	0.833	0.437 to 0.992
		$CA25^+DNA^+$ EV	1.000	1.000 to 1.000	0.535	1.000	0.845 to 1.000	1.000	0.610 to 1.000