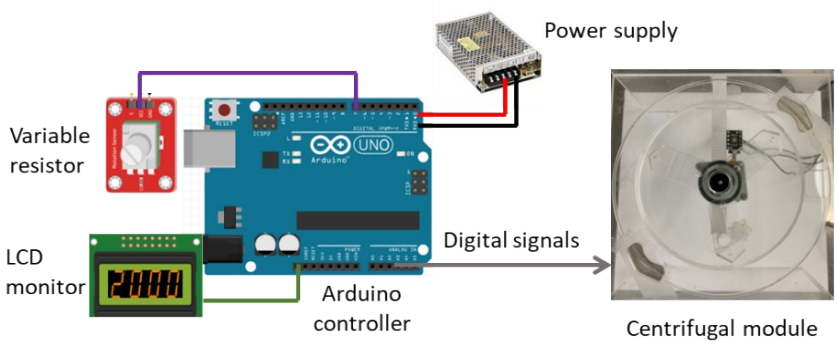
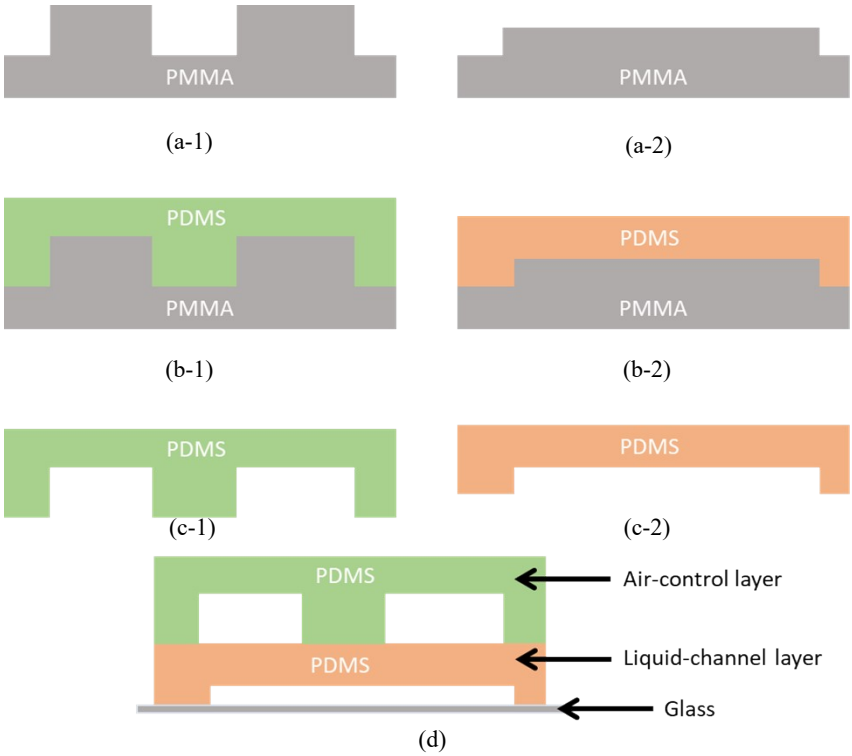
Electronic Supplementary Material (ESI) for Lab on a Chip.

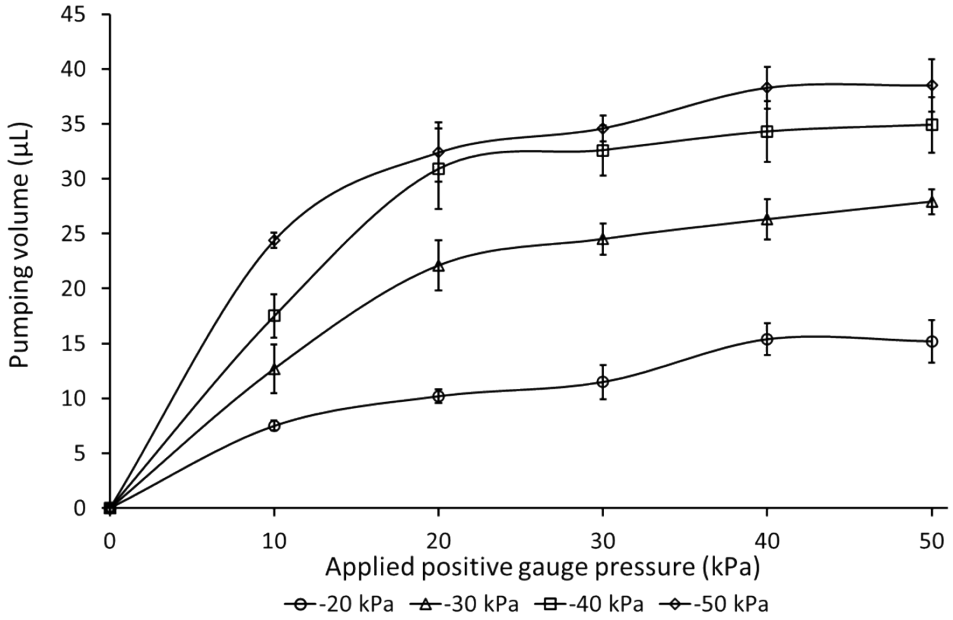
Supplementary Information



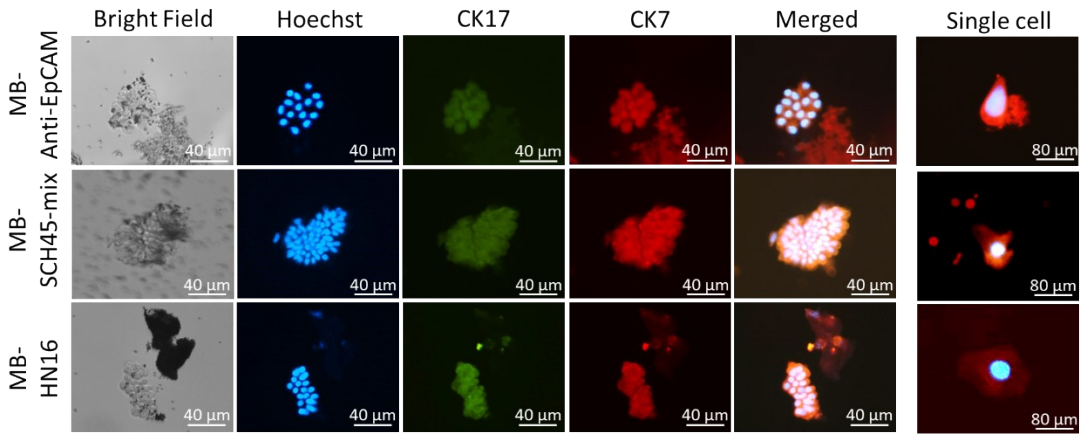
Supplementary Figure S1. A schematic diagram of the centrifugal module for sample pretreatment. The system utilized an Arduino microcontroller that sent digital signals to control the centrifugal force (in RPM). These digital signals were then transmitted to a variable resistance component, which initiated the centrifugation process. A variable resistor was utilized to control the rotational speed during the experiment. An LCD monitor was employed to verify that the target level of 2,000 RPMs was reached.



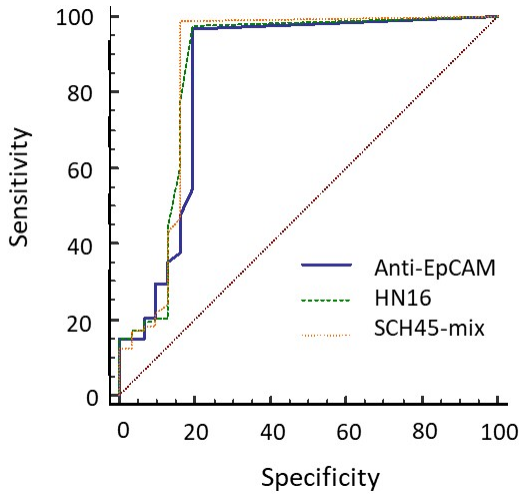
Supplementary Figure S2. The microfabrication process of the developed microfluidic modules. Panels (a-1) and (a-2) illustrated the PMMA micromachining process, which involved microfabrication of the air-control and liquid-channel layers by using a computer-numerical-control (CNC) drilling machine. Panels (b-1) and (b-2) demonstrated the PDMS casting process for the air-control and liquid-channel layers, respectively. They were both baked at 80°C for 2.5 hr. Panels (c-1) and (c-2) depicted the PDMS replication process for the air-control and liquid-channel layers, respectively. Finally, panel (d) showcased the PDMS+glass bonding step for creating the pneumatic microfluidic modules.



Supplementary Figure S3. Characterization of the micropump. The relationship between the volume of a single pumping iteration at various positive gauge pressures (0, 10, 20, 30, 40, & 50 kPa) cooperates with various negative gauge pressures (-20, -30, -40, & -50 kPa). Under the optimal conditions of 40/-50kPa, the target volume of 40 µL was transferred with high precision (std. dev.=1.9 µL; n=3).



Supplementary Figure S4. Images of ETCs isolated by affinity reagent-coated beads on-chip under a fluorescent microscope. Bright field images showed tumor cells and magnetic beads. ETCs were isolated by Anti-EpCAM (top row), SCH45-mix (middle row), HN16 (bottom row) followed by immunostaining with CCA biomarkers: CK17 (green) and CK7 (red; merged, CK17+CK7 images are also shown.). Hoechst (blue): nuclear staining.



Supplementary Figure S5. The receiver operating characteristic (ROC) curve was used to evaluate the performance of three different magnetic beads (SCH45-mix, anti-EpCAM, & HN16) in capturing CCA tumor cells from 156 clinical bile samples. The area under the curve (AUC) was calculated for each type of affinity reagent-coated magnetic bead. Importantly, the results showed that the *p*-values for all affinity reagents were less than 0.05 ($p<0.05$).

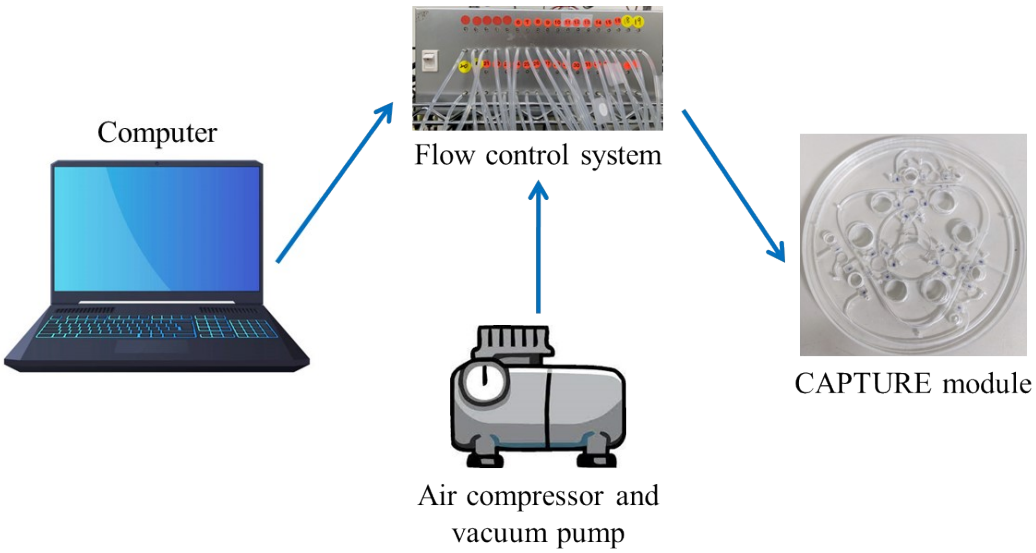


Figure S6: Illustration of the CAPTURE experimental setup. With one flow control system, and air compressor and vacuum, the micro components on the CAPTURE module could be operated by using a computer.

Supplementary Table S1. The on-chip protocol for sample pretreatment and the CAPTURE assay on the integrated microfluidic system. MBs=magnetic beads.

Procedure	Steps	Reagents	Components	Conditions
1. Reagent- and sample-loading	1. Load all reagents into their respective chambers	PBS Affinity reagents CK7 & CK17 (1^{st}) DyLight 488 & DyLight 594 (2^{nd}) Hoechst	Chamber S Chamber P Chamber A Chamber B Chamber F	Positive gauge pressure: 40 kPa Negative gauge pressure: -50 kPa Centrifugation: 2000 RPM for 20 min
2. Cell isolation (centrifugal module)	1. Load bile sample 2. Centrifuge for 20 min @ 1,500 RPM 3. Collect pellets 4. Transfer to CAPTURE module	1 mL clinical sample 1 mL clinical sample 1 mL clinical sample	Chamber P Chamber P Chamber P	Positive gauge pressure: 40 kPa Negative gauge pressure: -50 kPa
3. CCA ETC capture (CAPTURE module)	1. Re-suspend pellets in 1X PBS 2. Mix with MBs 3. Capture ETCs (30 min) 4. Wash twice	0.4 mL 1X PBS 0.4 mL 1X PBS+4 µL MBs anti-EpCAM/SCH 45/HN16 0.4 mL 1X PBS x 2	Chamber P Chamber P Chamber S Chambers P/S/W	Positive gauge pressure: 40 kPa Negative gauge pressure: -50 kPa
4. CCA ETC immunostaining (CAPTURE module)	1. Mix with 1^{st} antibody 2. Wash twice 3. Mix with 2^{nd} antibody 4. Wash twice	0.3 mL MBs+1 µL 1^{st} antibody (CK7/CK17) 0.4 mL 1X PBS x 2 0.3 mL MBs+1 µL 2^{nd} antibody 0.4 mL 1X PBS x 2	Chamber A/B Chamber F	Positive gauge pressure: 40 kPa Negative gauge pressure: -50 kPa
5. Nuclei staining	1. Transfer liquid to chamber F 2. Observe ETCs	0.4 mL beads+1^{st},2^{nd}antibody mixture with 1 µL Hoechst		Positive gauge pressure: 40 kPa Negative gauge pressure: -50 kPa

Supplementary Table S2. Average number of exfoliated tumor cells detected in patients

Patients	Disease	MB-SCH45 mix		MB-EpCAM		MB-HN16	
		P	N	P	N	P	N
1	Cholangiocarcinoma	2	0	3	0	1	0
2	Recurrent symptomatic gallstones with CBD stones with obstructive jaundice	0	0	0	0	0	0
3	GB stone	0	0	0	0	0	0
4	CBD wall thickening	0	0	0	0	0	0
5	GB polyp s/p LC	3	0	15	0	7	0
6	Cholangiocarcinoma	56	0	78	0	7	0
7	Cholangiocarcinoma	8	0	17	0	15	0
8	GB stone	0	0	22	3	2	0
9	distal common bile duct stricture complicated with obstructive jaundice	3	0	25	1	2	0
10	Cholangiocarcinoma (after chemotherapy)	0	0	0	0	0	0
11	Intrahepatic cholangiocarcinoma (ICCA) (after chemotherapy)	0	0	0	0	0	0
12	Cholangiocarcinoma (after chemotherapy)	0	0	0	0	0	0
13	GB polyp and stone → LC	0	0	0	0	0	0
14	metastasis or primary CBD or pancreatic malignancy (true negative)	0	0	0	0	0	0
15	Acute cholecystitis	0	0	0	0	0	0
16	GB stones with chronic cholecystitis, s/p LC	0	0	0	0	0	0
17	S5 tumor, suspect HCC, s/p Lap. partial hepatectomy (true negative)	0	0	0	0	0	0

18	Symptomatic GB stones, s/p laparoscopic cholecystectomy	0	0	0	0	0	0
19	Symptomatic GB stones, s/p laparoscopic cholecystectomy	0	0	0	0	0	0
20	CBD stone	0	0	0	0	0	0
21	Gallbladder adenocarcinoma	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
22	Ampullary tumor with distal common bile duct involvement	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
23	Favor Klatskin tumor with obstructive jaundice	2	0	10	0	3	0
24	IgG4 cholangiopathy	0	0	0	0	0	0
25	Favor cholangiocarcinoma	0	0	1	0	34	0
26	Suspected cholangiocarcinoma	121	0	214	3	70	0
Average ETCs per 1 mL of bile for true-positive cases while excluding the cases with TNTC (too numerous to count): MB-SCH45 mix: 32. MB-EpCAM: 54. MB-HN16: 22.							

Supplementary Table S3. Results from severe inflammation cases obtained through single-blind analysis with clinical bile samples analyzed with the CAPTURE assay. These findings highlight the significant association between severe inflammation and the progression of CCA tumors. Therefore, it was essential to take this factor into consideration when evaluating patients with worsening cholestasis. CBD=Common bile duct. GB=gallbladder. LC= Laparoscopic cholecystectomy. s/p= Status post. P: CK17+/CK7+/Hoechst+ cells in bile tests; N: CK17-/CK7-/Hoechst+ cells in bile tests.

Patient #	**Clinical information**	**Bile**					
		MB-SCH45 mix		**MB-Anti-EpCAM**		**MB-HN16**	
		P	***N***	***P***	***N***	***P***	***N***
1	GB polyps	36	0	26	1	23	0
2	Distal CBD stone	10	0	6	1	32	0
3	GB polyp	4	0	31	2	3	0
4	Distal CBD stone with pancreatic swelling	163	0	70	2	21	0
5	Acute cholecystitis	163	0	326	0	248	0
6	GB stone and polyp	20	0	34	0	13	0
7	GB stones with sludge & mild CBD/IHD dilation	0	0	2	0	0	0
8	GB stone	5	0	12	1	13	0
9	GB polyp s/p LC	3	0	15	0	7	0

Supplementary Table S4. Capture rates of each affinity reagent and for mixing three affinity reagents

Capture rate in %

	SCH45-mix	HN16	Anti-EpCAM	Anti-EpCAM +HN16 (1:1)	Anti-EpCAM +SCH45-mix (1:1)	HN16 +SCH45-mix (1:1)	Anti-EpCAM +HN16+SCH45-mix (1:1:1)
MMNK-1 (normal cells)	20.2±4.0	18.8±2.0	25.4±5.0	18.6±4.0	29.7±11.0	29.1±16.0	24.5±14.0
HuCCT-1 (tumor cells)	64.5±7.0	71.8±6.0	66.5±5.0	79.6±6.0	76.4±14.0	73.3±8.0	62.1±5.0

The on-chip experimental process of the CAPTURE module is as follows.

(1) Transfer the pellet separately to the CAPTURE module, which contains three chambers (chamber P), each with a different affinity reagent (total of three chamber P).

(2) Transfer the affinity reagents in chamber P along with the pellet to the micromixer to isolate ETCs.

(3) After the reaction is complete, wash away unnecessary impurities with PBS and remove them from chamber W. Introduce the primary antibody into chamber A for immunostaining reaction with ETCs.

(4) After the reaction is complete, wash away unnecessary impurities with PBS and remove them from chamber W. Introduce the secondary antibody into chamber B for strengthening the immunostaining signal with ETCs.

(5) After the reaction is complete, transfer the liquid to chamber F and allow Hoechst to react. Then, move the CAPTURE module to a fluorescence microscope for observation.

(6) The entire on-chip experimental process takes a total of 170 minutes.