Supplementary Information

Dual-patterned Immunofiltration (DIF) Device for the Rapid Efficient Negative Selection of Heterogeneous Circulating Tumor Cells

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S1. An Overview of the Overall Process

The verification of the dual-immunoflitration (DIF) devices was proceeded as follows.
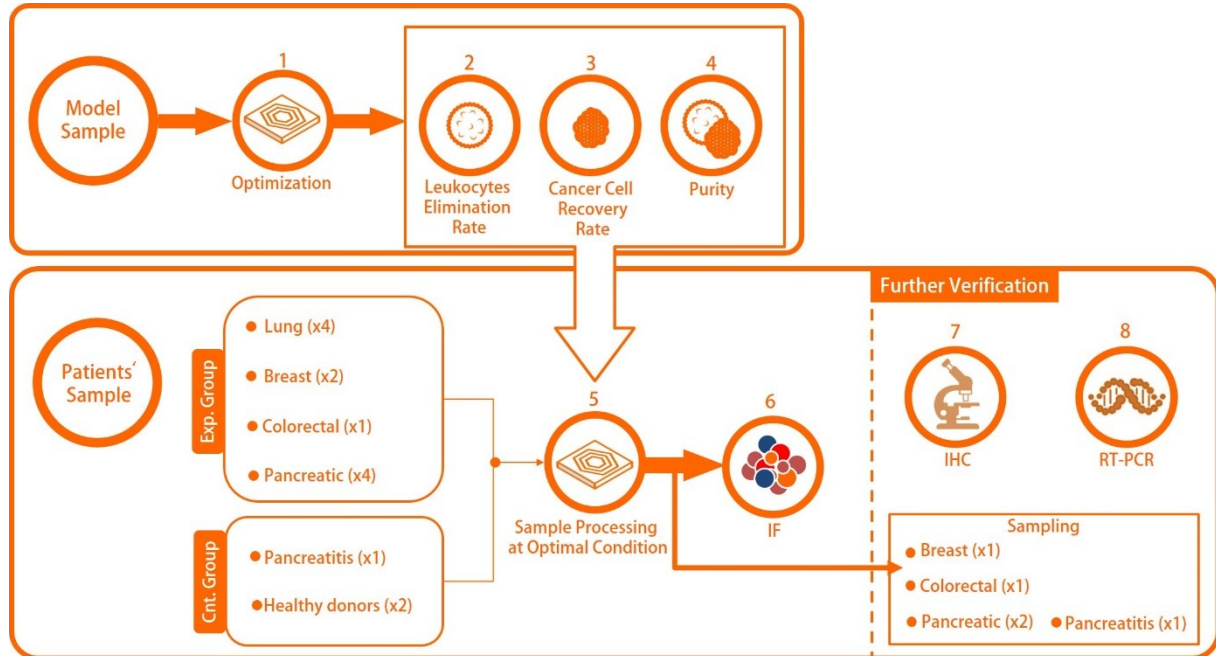
Figure S1. An overview of the overall experiment using DIF devices

Briefly, the optimal condition for the fabricated DIF devices was optimized [1] and evaluated on the basis of leukocyte elimination rate [2], cancer cell recovery rate [3], and purity [4]. After optimization of DIF using model samples, 11 pateints' samples (lung, breast, colorectal, and pancreatic) and 3 control samples (pancreatitis, helthy donors) was processed by DIF at an aforementioned condition [5]. Then, the processed samples were cytospinned and verified using Immunofluorescence (IF) analysis through complete enumeration [6]. Meanwhile, the half of the processed sample from 4 patients' samples (breast, colorectal, and pancreatic) and 1 control sample (pancreatitis) was also prepared for further verification, immunohistochemistry [7] and RT-PCR [8]. On the subject of further verification, it is hard to examine all the sample set; thus, we did a stratified sampling by picking one-third of them, including 3 different cancer patients' samples and 1 control sample.

S2. Evaluation Criteria for the DIF devices
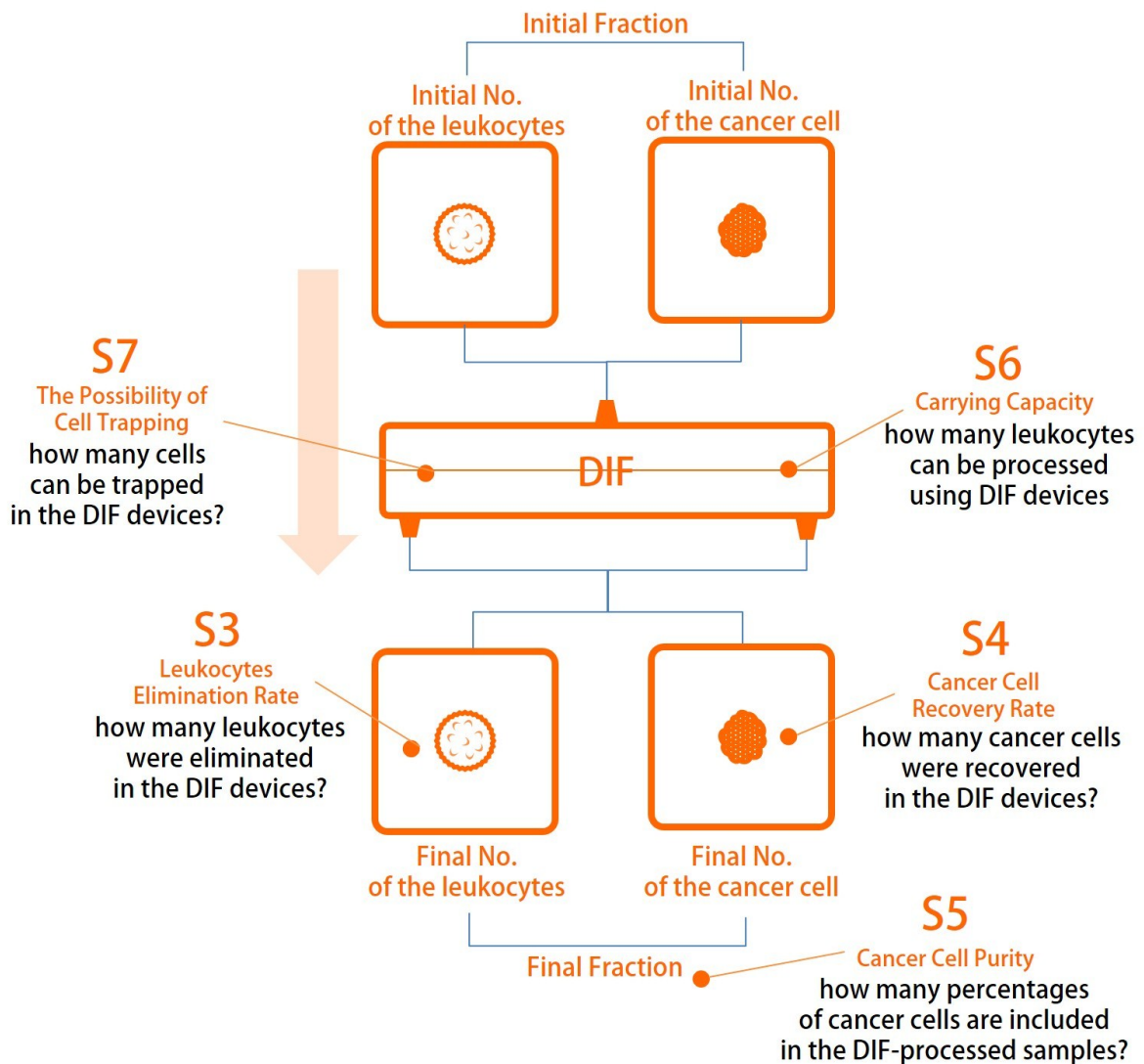
Figure S2. Evaluation criteria for performance of the DIF devices

To date, a number of articles have been reported negative selection strategy in circulating tumor cell research, and these articles have introduced several criteria for the evaluation of the results; thus, we utilized these common criteria for the evaluation of the DIF devices: leukocytes elimination rate (S3), cancer cell recovery rate (S4), cancer cell purity (S5). In addition to that, we also introduced two evaluation criteria, carrying capacty (S6) and the possibility of cell trapping (S7), for the verification from a device-focused the view.

S3. Leukocytes Elimination Rate

In the previous literatures related to negative selection, the most mentioned criteria for evaluating leukocytes elimination was leukocytes elimination rate. It is defined as follow:

$$Leukocyte\ Elimination\ (\%) = \frac{(No.\ of\ the\ eliminated\ leukocyte)}{(Initial\ No.\ of\ the\ leukocytes)} \times 100\ (\%)$$

Sometimes it is described as a term called enrichment yield due to its simplicity. This factor is simply calculated by dividing the initial number of leukocytes to the final number of leukocytes. It seems not much different from leukocytes elimination rate; it is useful in helping us to discriminate a small difference in leukocytes elimination rate. For example, 99.90 % and 99.99 % of leukocytes elimination rate can be converted to 1,000 and 10,000 of enrichment yield, respectively). Based on the leukocytes elimination rate, it is easy to underestimate the diffrence between them whereas the diffrence in enrichment yield is quite evident. The enrichment yield of the the present devices (NIFs, SIFs, and DIFs) were 5.11, 5.81, and 75.51, respectively. This means that DIF devices showed 14 times higher leukocytes elimination efficiency than others.

$$Enrichment\ Yield\ (\%) = \frac{(Initial\ No.\ of\ the\ leukocytes)}{(No.\ of\ the\ recovered\ leukocyte)} \times 100\ (\%)$$

Meanwhile, some researchers prefer to use the term $\log_{10}$ enrichiment in place of enrichment yield because the latter is more helpful in understanding the performances. For example, 1.0 of $\log_{10}$ enrichiment means one out in ten leukocytes still remain after filtration with the present devices. Likewise, 2.0 of $\log_{10}$ enrichiment means one in a hundred leukocytes remain in the processed sample because ninety-nine in a hundred leukocytes might be eliminated. Given that one mililiter of human blood contains approximately 5 million of leukocytes, this factor cannot

be allowed to exceed 6.0 (at that moment, only 5 leukocytes are included in the processed sample). The $\log_{10}$ enrichiment of the the present devices (NIFs, SIFs, and DIFs) were 0.69, 0.76, and 1.85, respectively.

$$log10\ enrichiment = log\frac{(Initial\ No.\ of\ the\ Cells\ \times Fraction\ of\ the\ leukocytes)}{(Final\ No.\ of\ the\ Cells\ \times Fraction\ of\ the\ leukocytes)}$$

The following is a comparative table of the present devices regarding to the three terms mentioned above. The grey-shade column represents the optimal condition applied to further experiments using patients' samples.

Table S1. Leukocytes elimination rate, enrichment yield, and $\log_{10}$ enrichiment of the processed sample using by the present devices (NIFs, SIFs, and DIFS)

Device Type	NIFs	SIFs	DIFs		
Flow-rate	1.0 ml/hr	1.0 ml/hr	0.5 ml/hr	1.0 ml/hr	1.5 ml/hr
Leukocytes Elimination Rate (%)	79.72 ± 4.99	82.32 ± 3.73	97.08 ± 1.60	**97.07 ± 1.14**	81.73± 7.05
Enrichment Yield	5.11	5.81	45.12	**75.51**	6.19
$\log_{10}$ Enrichiment	0.69	0.76	1.56	**1.85**	0.76

S4. Cancer Cell Recovery Rate

Two kinds of terms have been introduced as a way of explaining the possibility of loss of cancer cells: cancer cell recovery rate and non-specific binding rate. Basically, these two terms are derived form the identical concept; the former one is the inverse form of the latter one. Therefore, if the former one is 50 %, the latter one will be presumed to be 50 %. As we mentioned in the manuscript, we decided to use this term in combination with the latter one for highlighting the unintentional loss of CTC-like cells in the aspect of device performance.

$$Cancer\ Cell\ Recovery\ Rate\ (\%) = \frac{(Final\ No.\ of\ the\ Cancer\ Cells)}{(Initial\ No.\ of\ the\ Cancer\ Cells)} \times 100\ (\%)$$

$$Nonspecific\ Binding\ Rate\ (\%) = \frac{(Initial\ No.\ of\ the\ Cancer\ Cells)}{(Final\ No.\ of\ the\ Cancer\ Cells)} \times 100\ (\%)$$

The following is a comparative table of the present devices regarding to the two terms mentioned above. The grey-shade column represents the optimal condition applied to further experiments using patients' samples.

Table S2. Cancer cell recovery rate and nonspecific binding rate
of the processed sample using by the present devices (NIFs, SIFs, and DIFS)

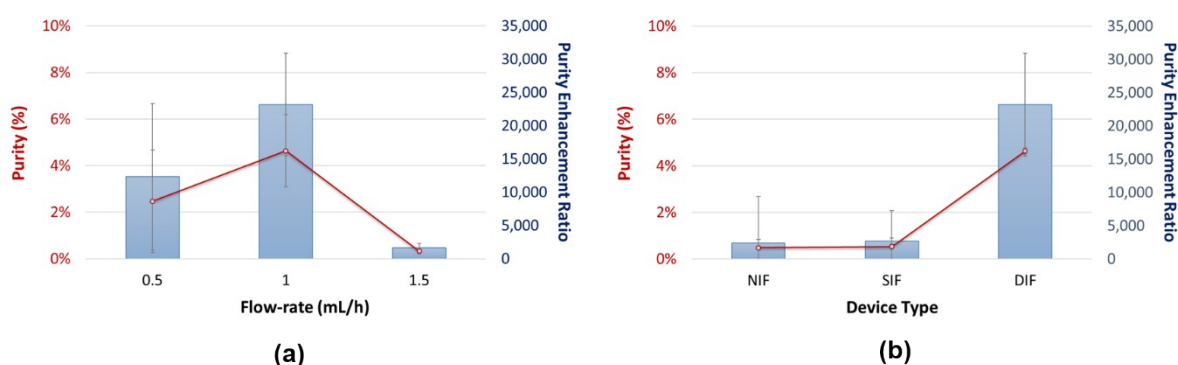| Device Type	NIFs	SIFs	DIFs		
Flow-rate	1.0 ml/hr	1.0 ml/hr	0.5 ml/hr	1.0 ml/hr	1.5 ml/hr
Cancer Cell Recovery Rate (%)	94.85 ± 1.29	92.21 ± 1.39	82.02 ± 3.20	**91.01 ± 4.77**	89.94 ± 6.28
Nonspecific Binding Rate (%)	5.15	7.88	17.98	**8.99**	10.06

S5. Cancer Cell Purity

Cancer cell purity means the ratio between the leukocyte and the cancer cell in the prossessed sample. It is calculated as follow:

$$Cancer\ Cell\ Purity\ (\%) = \frac{(Final\ No.\ of\ the\ Cancer\ Cells)}{(Final\ No.\ of\ the\ Cells)} \times 100\ (\%)$$

However, this factor is a relative value; it can be estimated quite diffrently by the initial amount of the leukocyte and the cancer cells. If the researcher use the model sample containing small amount of leukocyte (less than 10^5) with a considerable number of cancer cells (more than 10^3), this factor can easily be exaggerated. Therefore, some researchers prefer to use the term purity enhancement ratio. Because it reflects the initial ratio between the leukocyte and the cancer cell, it is considered as more reliable concept.

Figure S3. The performance of the DIF devices:
purity (red line) and purity enhancement ratio (blue bar) as a function of
(a) the flow rates (0.5 mL/h, 1.0 mL/h, and 1.5 mL/h), (b) design (NIF, SIF and DIF)

Table S3. Cancer cell purity and purity enhancement ratio of the processed sample
using by the present devices (NIFs, SIFs, and DIFS)

| Device Type	NIFs	SIFs	DIFs		
Flow-rate	1.0 ml/hr	1.0 ml/hr	0.5 ml/hr	1.0 ml/hr	1.5 ml/hr
Cancer Cell Purity (%)	0.48 ± 1.05	0.53 ± 0.96	2.47 ± 2.20	**4.64 ± 1.54**	0.33 ± 0.13
Purity Enhancement Ratio	2414	2674	12,334	**23,188**	1654

S6. Carrying Capacity

The carrying capacity is originally a ecology term referring the maximum number of individuals of a given species that an area's resources can sustain indefinitely without significantly depleting or degrading those resources. Later, the meaning have been expanded to include the containability of a particular system: the number or quantity of people or things that can be conveyed or held by a vehicle or container. In the field of evironmental engineering, sometimes it means the amount of the eliminated contaminant by using purification system or sewage disposal facilities. In our case, leukocytes were considered as a kind of contaminant; thus, we borrowed the term from those fields to explain the maximum containability of the present device. It is defined as follow: the amount of leukocytes that can be processed using the present device. As mentioned in the manuscript, it was roughly estimated to 16.7 ± 1.5 million.

S7. The Possibility of Cell Trapping

Due to the uniqely designed top and bottom patterns, we archived high leukocytes elimination rate and cancer cell recovery rate; these improvements also had the possibility to cause potential drawback of the trapped (clogged or omitted) cells between the patterns. Honestly, it is hard to enumerate the trapped cells since most of them were instantly lost when both patterns were decoupled. If it is even possible, some of them are not distinguished from the specifically isolated cells and nonspecifically adsorbed cells. That is the reason why we had to estimate these cells depending on the indirect calculation of the remined cancer cells.


As it can be seen in Fig. S1-(c) and Fig. S2, some cancer cells were found inside corner of the engraved patterns. We basically assumed that they were nonspecifically-adsorbed; but, they could be the trapped cells owing to structural design. The only possible way to estimate the amount of them is comparing with other device types, which do not have the complicated patterns to cause trapping. As mentioned in S4, nonspecific binding of the cancer cells slightly increased on the DIFs (8.99 %) compared to NIFs (5.15 %) and SIFs (7.88 %). We hypothesize that these differences (3.74 % of DIFs-NIFs difference and 1.11% of DIFs-SIFs difference) might be partly or mainly caused by structural design since the possiblity of cell trapping on the NIF devices is extremly low. Also, the increased non-spcific binding rate on the DIF devices at a lower flow rate (17.89 % at 0.5 ml/hr) supports the hypothesis; if there were chances of cell trapping inside of the patterns, it might be rapidly elevated at a lower flow rate.
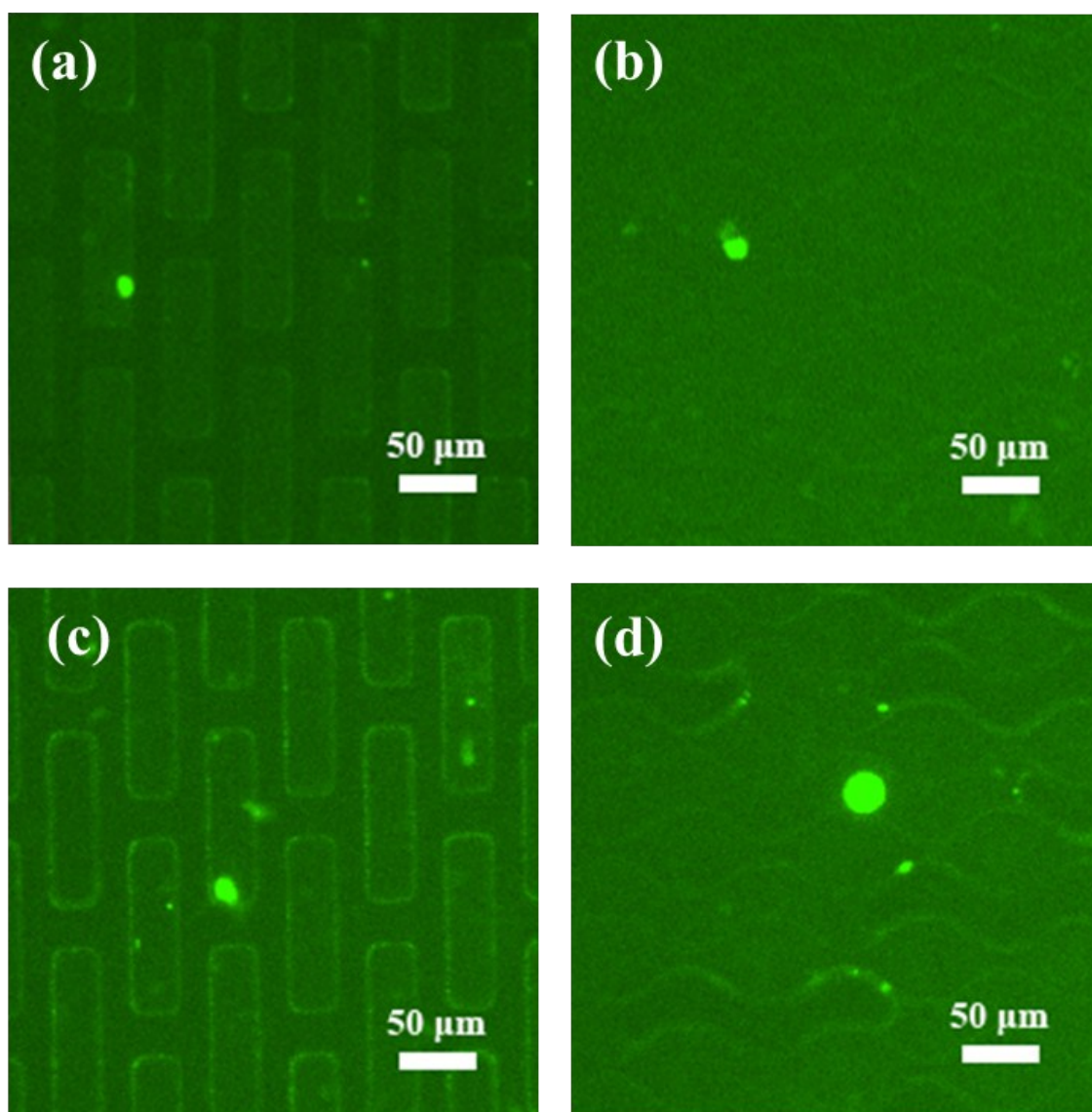
Figure S4. Confirmation of the non-specifically adsorbed cancer cells (CellTracker green labelled A549) on the DIF device using fluorescence microscopy: (a, c) top layer; (b, d) bottom layer.
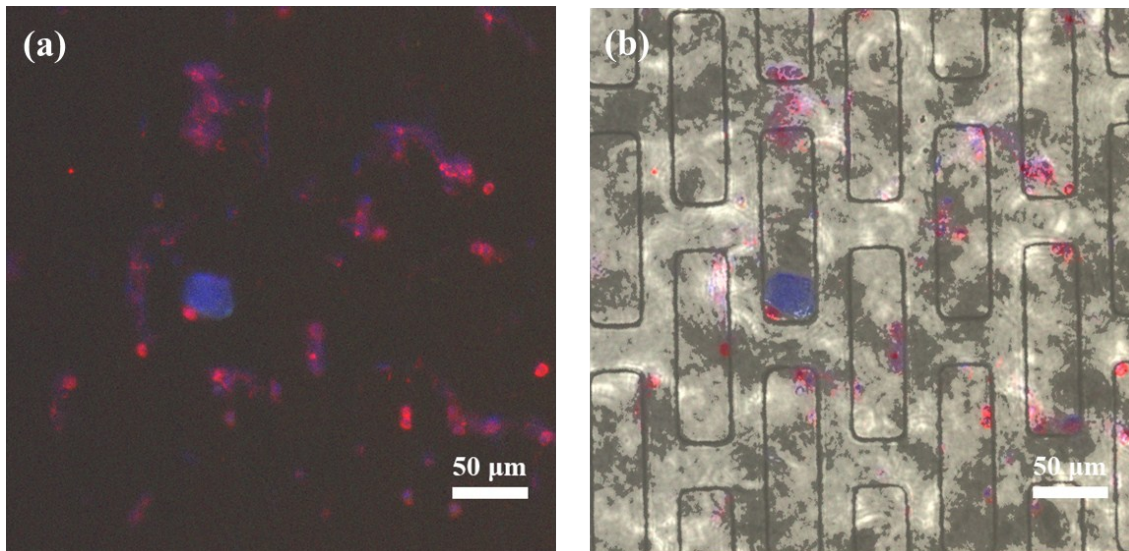
Figure S5. (a) Merged image of non-specifically adsorbed CTC on the top layer of DIF device after immunofluorescence staining (DAPI: blue, CD45: red, and CK: green); (b) bright field image added on figure (a).
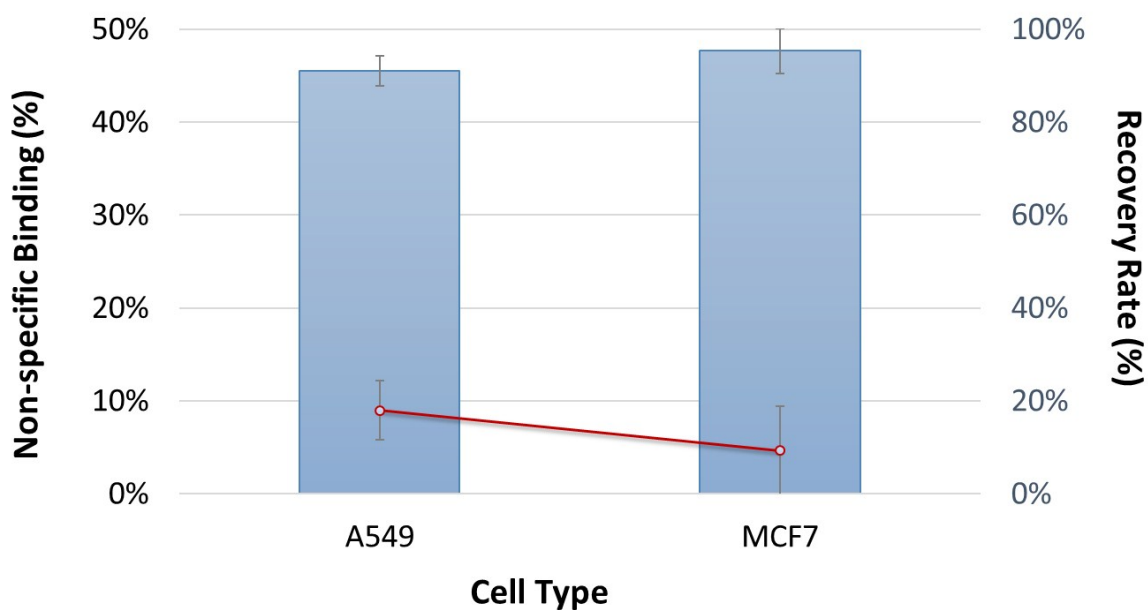
Figure S6. Recovery rate (red line) and non-specific adsorption (blue bar) of the spiked cancer cells in accordance with the type of the spiked cells. The experiment was performed with DIF devices at the optimal condition (1 ml of sample volume; 1 ml/he of rate, 100 of each spiked cells). All trial was repeated 3 times.