SUPPORTING INFORMATION

Multiplexed Magnetic Labeling Amplification Using Oligonucleotide Hybridization

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METHODS

All chemicals and solvents were purchased from commercial suppliers and used as received. Dimethylformamide, dimethyl sulfoxide, sodium hydroxide, hydrochloric acid, sodium bicarbonate, formaldehyde solution (37%), penicillin and streptomycin solution, L-glutamine were purchased from Sigma-Aldrich. Bovine serum albumin (BSA), 10x phosphate buffered saline (PBS) liquid concentrate, ethylenediaminetetraacetic acid (EDTA), sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC), *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), dithiothreitol (DTT) were purchased from Thermo Fisher Scientific. VivoTag 680 (VT680) was purchased from PerkinElmer. Fluorescein isothiocyanate (FITC), Alexa Fluor 568 (AF568), TO-PRO-1 iodide were purchased from Invitrogen. Dulbecco's Modified Eagle Medium (DMEM), Trypsin/EDTA, and Hank's balanced salt solution (HBSS) were purchased from Mediatech. Fetal bovine serum (FBS) was purchased from ISC BioExpress.

List of oligonucleotides. The oligonucleotides were purchased from Integrated DNA Technologies. The list of oligonucleotides used in the experiments are summarized below.

DNA1: 5'-thiol modifier-CGCATTCAGGAT-3' for conjugation to magnetic nanoprobes and antibodies, 5'-biotin-CGCATTCAGGAT-3' for SPR immobilization
DNA2: 5'-TCTCAACTCGTA-thiol modifier-3'
DNA3: 5'-thiol modifier-CTTCACTCATAT-3'
DNA4: 5'-AGTTCCGTGTTA-thiol modifier -3'
DNA5: 5'-thiol modifier-ATGTGGGCAGTC-3'
DNA6: 5'-GTCATTTAGCAT-thiol modifier -3'
DNA7: 5'-thiol modifier-ATCCTGAATGCG-3'

Oligonucleotide linkers (*L*-DNA):
Linker-1: 5'-TACGAGTTGAGAATCCTGAATGCG-3' for DNA1 and DNA2
Linker-2: 5'-TAACACGGAACTATATGAGTGAAG-3' for DNA3 and DNA4
Linker-3: 5'-ATGCTAAATGACGACTGCCCACAT-3' for DNA5 and DNA6

Morpholino oligonucleotides for Linker-1 were purchased from Gene Tools, LLC.

Synthesis of magnetic nanoprobes (MNPs). Cross-linked dextran-coated iron oxide particles (CLIO-47) were used for the magnetic labeling of cells. These particles are approximately 30 nm in hydrodynamic diameter (3 nm iron oxide core) and have an r_2 value of 70 $mM^{-1}[Fe]\ s^{-1}$. The

synthesis of amine-terminated probes and their conjugation with fluorescent molecules (FITC, AF568, and VT680) were both done using a previously described protocol.[1] Each fluorescent conjugate had 5-8 dye molecules and 90 amine groups for further conjugation. To attach oligonucleotides to the probe, MNPs (0.25 mg) were first mixed with sulfo-SMCC (90 μg) in 0.625 mL PBS solution (pH 7.2) for 3 hours. Concurrent to this, thiol-modified oligonucleotides (46.6 nmol) were mixed with DTT (2.875 mg) in 0.1 mL PBS (1 mM EDTA, pH 8) for 2 hours. The reduced oligonucleotides were then purified using a NAP-5 column (GE Healthcare) and deionized water as the eluent solution. Absorbance measurements were used to determine the fractions containing the reduced oligonucleotides and the bicinchoninic (BCA) protein assay solution (Thermo Fisher Scientific) were used to determine the fractions containing the DTT. MNP-SMCCs were subsequently purified using membrane filtration (Millipore Amicon, MWCO 30,000) and Sephadex G-50 (GE Healthcare) with PBS as the eluent buffer. The purified maleimide-activated MNPs were eventually mixed with the reduced oligonucleotides in 2 mL PBS solution (1 mM EDTA, pH 7.2) and the reaction proceeded overnight at 4°C. After this incubation, the conjugates were purified using membrane filtration and Sephadex G-100 with PBS as the eluent buffer. Conjugation with FAM-modified oligonucleotides was used to confirm that approximately 50 oligonucleotides were conjugated to each fluorescent MNP-DNA probe.

Surface plasmon resonance (SPR) measurements. The measurements were performed on the Biacore T100 instrument using a carboxymethylated dextran chip pre-immobilized with streptavidin (SA sensor chip). The P-20 surfactant and the SA chip were obtained from GE Healthcare.

The sensor chip surface on flow cell 4 (Fc4) was initially activated with three 60-second injections (10 μL/min) of regeneration solution (50 mM NaOH, 1 M NaCl). These were followed by pulse injections of biotinylated DNA1 oligonucleotides (50 nM in PBS-P) so as to obtain a capture level of 120 response units (RU). Flow cell 3 (Fc3) was used as a reference surface with captured non-complementary oligonucleotides. Binding was measured at 25°C. PBS (pH 7.4) containing 0.005% P-20 surfactant (PBS-P) was used as the dilution and running buffer. Analytes were characterized by measuring binding at a range of concentrations (1:2 dilution series in duplicate) using a single cycle approach, where each analyte was injected with increasing concentrations in a single cycle. The surface was not regenerated between injections. Single cycle runs with 5 concentrations per cycle were selected in order to allow for 80 second analyte contact times (association phase) at 30 μL/minute, and a subsequent 120 second dissociation phase. After each single cycle run the surface was regenerated with a 30 second injection (60 μL/min) of 50 mM NaOH in 1 M NaCl. To measure binding between DNA2 and *L*-DNA, the surface was initially treated with Linker-1. This was accomplished by injecting 500 nM Linker-1 over the analysis surface for 240 seconds at 10 μL/minute prior to single cycle injections of analyte. The resulting binding curves were double reference subtracted and globally fitted to a one-to-one binding model.

The binding of MNP-DNA probes to oligonucleotide surfaces were then measured on the same sensor chip as described for free oligonucleotides. In order to prevent nonspecific binding to the

reference surface, MNP-DNA probes were treated with succinic anhydride to terminate the free amines on the surface coating. Binding was measured using the traditional multi-cycle analysis method. In a typical binding assay, *L*-DNA is captured by injecting 500 nM solution over the analysis surface for 240 seconds (10 μL/minute) prior to injection of the analyte. A 120 second association phase (20 μL/minute) is followed by a 120 second dissociation phase. At the end of each cycle, the surface is regenerated with a 30 second injection (60 μL/minute) of 50 mM NaOH in 1 M NaCl. This capture and regeneration with each cycle results in slightly differing oligonucleotide immobilization densities, and thus variable R_{max} for different MNP-DNA concentrations. MNP-DNA binding was assessed in duplicate as a function of probe concentration (based on 8000 Fe atoms/MNP) in PBS-P, increasing from 3.5 nM to 14 nM in 1:2 dilution. The resulting binding curves were double reference subtracted and locally fitted to a one-to-one binding model.

Multiple rounds of binding were assessed by alternating injections of *L*-DNA (500 nM) and complementary MNP-DNA probes (20 nM MNP-DNA1 or 20 nM MNP-DNA2) using the manual run protocol. The flow was set to 10 μL/minute over the Fc4-Fc3 flow path. To generate four rounds of MNP binding, multiple injections lasting 360 seconds each (to ensure binding saturation) were made in succession.

Antibody modifications. The following antibodies were used in the experiments: anti-HER2 antibodies (Trastuzumab; Genentech) for HER2/*neu* markers, anti-EpCAM (Human EpCAM/ TROP1 MAb, Mouse IgG 2a/b; R&D Systems) for EpCAM markers, anti-EGFR (Cetuximab; ImClone Systems) for EGFR markers. To attach oligonucleotides to the antibody, each antibody (0.25 mg) was mixed with sulfo-SMCC (36.4 μg, 50x molar excess) for 90 minutes at room temperature and purified with gel filtration using PBS (1 mM EDTA, pH 7.2). Thiol-modified oligonucleotides (23.3 nmol) were then reduced with DTT (1.44 mg) in 0.1 mL PBS (1 mM EDTA, pH 8) for 2 hours. The reduced oligonucleotides were purified using NAP-5 column, and deionized water as the eluent solution. The maleimide-activated antibody was mixed with the reduced oligonucleotides in 1 mL PBS solution (1 mM EDTA, pH 7.2). The reaction proceeded overnight at 4°C, after which the conjugates were purified using membrane filtration and gel filtration, with PBS as the eluent buffer. Based on absorbance measurements and the BCA protein assay, each antibody contained approximately 8 oligonucleotide strands.

To prepare MNP-mAbs (direct conjugate), MNPs (0.25 mg) were mixed with SMCC (90 μg) for 3 hours at room temperature. Meanwhile, the antibody (0.5 mg) was reduced with DTT (3x molar excess) in PBS (1 mM EDTA, pH 8) for 60 minutes. The purified MNP-SMCC was then mixed with the reduced antibodies overnight at 4°C and purified with Sephadex G-100 in PBS. To prepare MNP-mAbs (DNA conjugate), MNP-DNA2 (0.25 mg) was mixed with Linker-1 before purification. The conjugates were then mixed with mAb-DNA1 (0.25 mg) and purified with Sephadex G-100 in PBS.

Cell labeling and detection. SK-BR-3, MCF-7, MDA-MB-231, A431, HCT-116 human cancer cells and NIH/3T3 fibroblasts were cultured in DMEM medium, supplemented with fetal bovine

serum (FBS; 10%), penicillin and streptomycin (1%), and L-glutamine (1%). Cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. At confluence, the cells were washed, trypsinized and resuspended in PBS solution containing 2% FBS and 1% BSA (C-PBS). In a typical labeling experiment, 10^5 cells were incubated in C-PBS containing mAb-DNA (10 μg/mL) for 20 minutes at room temperature. Following aspiration and centrifugation, the cells were mixed with *L*-DNA (5 μM) for a further 20 minutes at room temperature. After purification, the cells were incubated with MNP-DNA probes (40 μg/mL). The cells were then fixed using BD Fix Buffer (BD Biosciences) for measurements. Control samples were prepared in the same manner, but were incubated without the antibody conjugates. Flow cytometry measurements were performed using BD LSR II flow cytometer and mean fluorescence intensity was determined using FlowJo software. Changes in transverse relaxation rate (ΔR_2) were measured using the miniaturized nuclear magnetic resonance system previously reported.[2] The sample volume per measurement was 1 μl. We used Carr-Purcell-Meiboom-Gill pulse sequences with the following parameters, echo time (TE): 4 mseconds; repetition time (TR): 6 seconds; the number of 180° pulses per scan: 500; the number of scans: 8. All measurements were performed in triplicate and the data are presented as mean ± standard error. The measured ΔR_2 value was then converted to the cellular relaxivity Δr_2 (R_2 divided by cell concentration), a value which is proportional to the number of MNPs per cell. The expression level of the target marker was obtained as $\xi^{mAb} = \Delta r_2^{mAb}/\Delta r_2^{ø}$, where Δr_2^{mAb} and $\Delta r_2^{ø}$ are cellular relaxivities for marker-specific and control MNPs, respectively.

For the experiments depicted in Figure 2, 10^5 SK-BR-3 cells were incubated in C-PBS solution containing either anti-HER2-DNA1 conjugates for HER2/*neu* targeting, or anti-EGFR-DNA1 conjugates for EGFR targeting (10 μg/mL). Following aspiration and centrifugation, C-PBS solution containing Linker-1 (5 μM) was then added to the cells. After purification, VT680-labeled MNP-DNA2 (40 μg/mL) was added, followed by alternate labeling with MNP-DNA1 and MNP-DNA2 probes, using Linker-1 strands in-between.

For the labeling of live cells and microscopy imaging, cells were grown to confluency in an 8-well chamber slide. After washing the cells with HBSS, the cells were labeled as described above, with the exception that cell culture medium was used instead of C-PBS. The cells were then fixed and permeabilized with BD Fix Buffer and BD Perm/Wash Buffer for 20 minutes before incubating in a solution of TO-PRO-1 iodide (1 μM in BD Perm/Wash Buffer) for 15 minutes.

Multiplexed detection. The following MNP-DNA probes were synthesized and used for multiplexed labeling and subsequent amplification. Note that DNA5 on MNPs are for amplification with MNP-DNA-6 (via Linker-3).

a) MNP-DNA2+5 modified with DNA2 and DNA5 for hybridization with anti-HER2-DNA1 (via Linker 1); targeting HER2/*neu*
b) MNP-DNA1+5 modified with DNA1 and DNA5 for hybridization with anti-EpCAM-DNA2 (via Linker 1); targeting EpCAM

c) MNP-DNA4+5 modified with DNA4 and DNA5 for hybridization with anti-EGFR-DNA6 (via Linker 3); targeting EGFR
d) MNP-DNA6 modified with DNA6 for hybridization with DNA5 (via Linker-3) and amplification of labeled markers
To synthesize the MNP-DNA probes with mixed oligonucleotides, a 50/50 ratio of the reduced oligonucleotides was added to the maleimide-activated MNPs.

The following mAb-DNA conjugates were synthesized and used for multiplexed labeling:
a) anti-HER2-DNA1 modified with DNA1 for HER2/*neu* targeting
b) anti-EpCAM-DNA2 modified with DNA2 for EpCAM targeting
c) anti-EGFR-DNA3 modified with DNA3 for EGFR targeting

For the multiplexed labeling and amplification experiments outlined in Figure S14, cells were initially labeled with all 3 mAb-DNA conjugates (10 μg/mL) and their respective *L*-DNA (5 μM). Following aspiration and centrifugation, the sample was labeled with MNP-DNA2+5 (40 μg/mL) before being transferred to the μNMR for measurement. This measurement yielded the HER2/*neu* expression level (ζ^{HER2}). The sample was then labeled with MNP-DNA1+5 and transferred for the second measurement, in which the difference between $\zeta^{HER2+EpCAM}$ and ζ^{HER2} yielded the EpCAM expression level (ζ^{EpCAM}). The sample was then labeled with MNP-DNA4+5 and transferred for the third measurement, in which the difference between $\zeta^{HER2+EpCAM+EGFR}$ and $\zeta^{HER2+EpCAM}$ yielded the EGFR expression level (ζ^{EGFR}). Finally, to amplify the overall signal from all labeled markers, the sample was labeled with MNP-DNA6 and measured. The final measurement represented the amplified triple marker combination (ζ^{Total}). To support the μNMR results, a separate experiment was also done using flow cytometry. The marker expression level that was measured and calculated from μNMR analysis, along with the amplified triple marker combination, is summarized in the heat map in Figure 3. The original measurements by the μNMR and flow cytometry are summarized in Figure S14.

SUPPORTING FIGURES

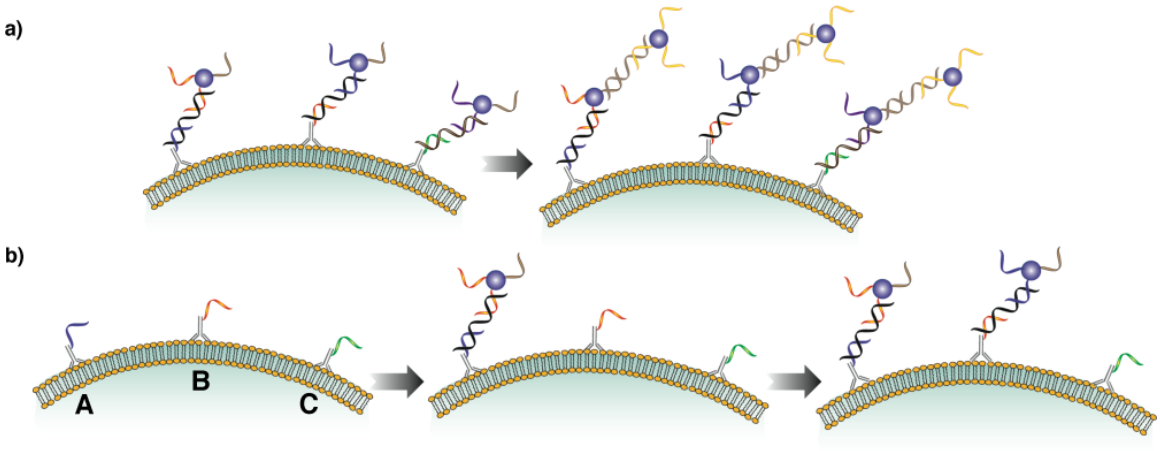
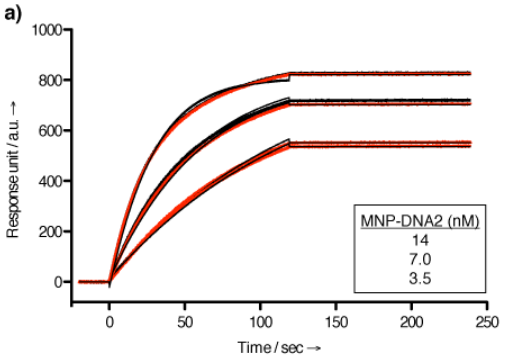
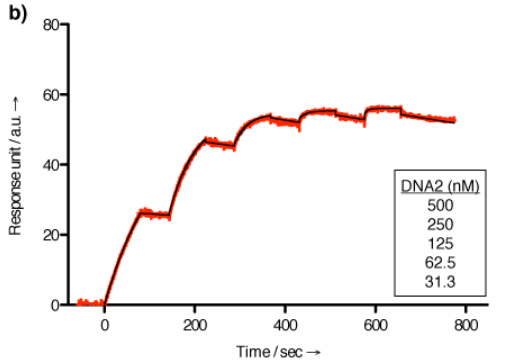


Figure S1. a) Amplified and b) multiplexed magnetic labeling of surface receptors.



k_a ($M^{-1}s^{-1}$)	SE (k_a)	k_d (s^{-1})	SE (k_d)	K_D (M)	chi^2 (RU^2)
2.85×10^6	3.4×10^3	1.58×10^{-6}	6.9×10^{-7}	5.56×10^{-13}	66.3



k_a ($M^{-1}s^{-1}$)	SE (k_a)	k_d (s^{-1})	SE (k_d)	K_D (M)	chi^2 (RU^2)
2.78×10^5	60	3.80×10^{-4}	3.5×10^{-6}	1.37×10^{-9}	0.20

Figure S2. Sensorgram of SPR experiments to determine binding dissociation constant K_D. Different concentrations of either a) MNP-DNA2 or b) free oligonucleotides DNA2 (reduced with DTT) were flowed over Linker-1/Biotin-DNA1 immobilized on the SPR sensor chip.

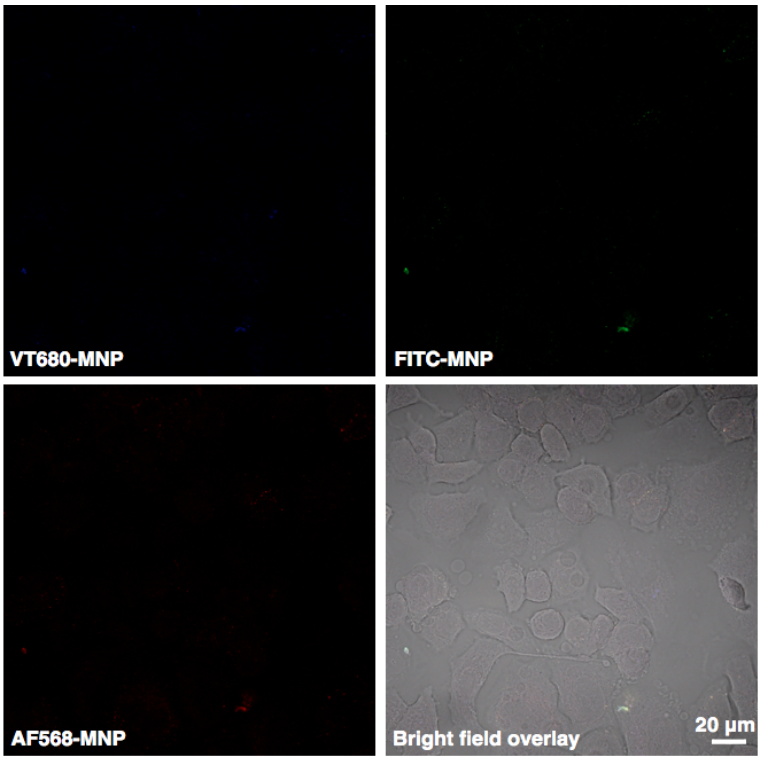


Figure S3. Confocal microscopy analysis. SK-BR-3 control cells were incubated with alternating rounds of fluorescent VT680-MNP-DNA2, FITC-MNP-DNA1, and AF568-MNP-DNA2 along with their respective *L*-DNA, but without the addition of HER2-DNA. The MNP-DNA probes showed low nonspecific binding within the nontargeted samples.

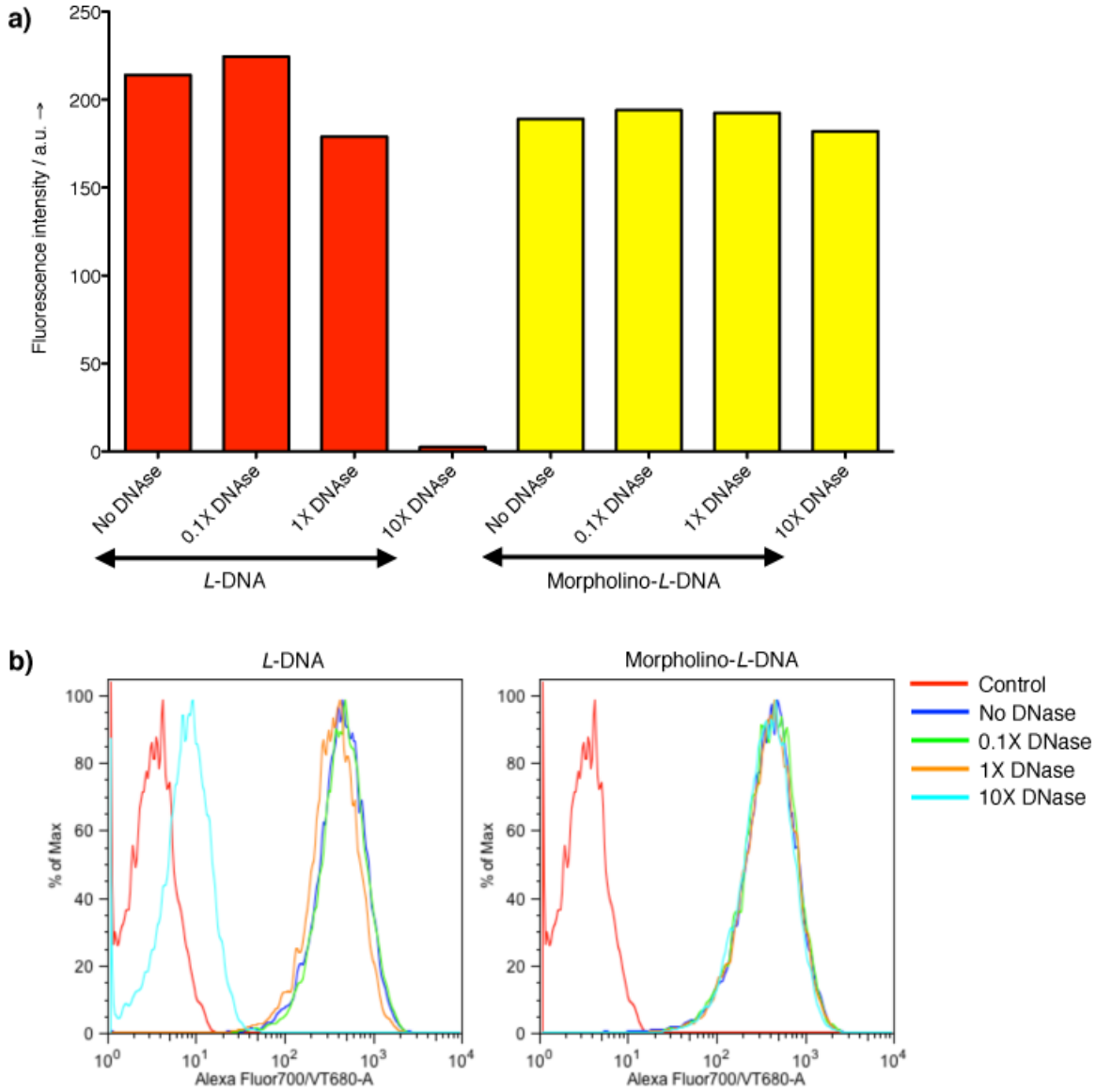


Figure S4. Nuclease degradation. a) The normalized fluorescence signal intensity. b) A histogram analysis of flow cytometry experiments demonstrating the effect of DNase on hybridization and magnetic labeling. Different molar ratios of DNase to oligonucleotides were mixed with the samples during the magnetic labeling process and incubated at 37°C to increase the enzymatic activity. *L*-DNA, composed of morpholino oligonucleotides, was resistant to degradation by DNase. Although the amount of DNase required to inhibit the labeling process is much higher than that of the standard condition, the morpholino oligonucleotides could be used as an alternative. For the control samples, mAb-DNA conjugates were omitted during the labeling process. Morpholino oligonucleotides for Linker-1 were purchased from Gene Tools, LLC.

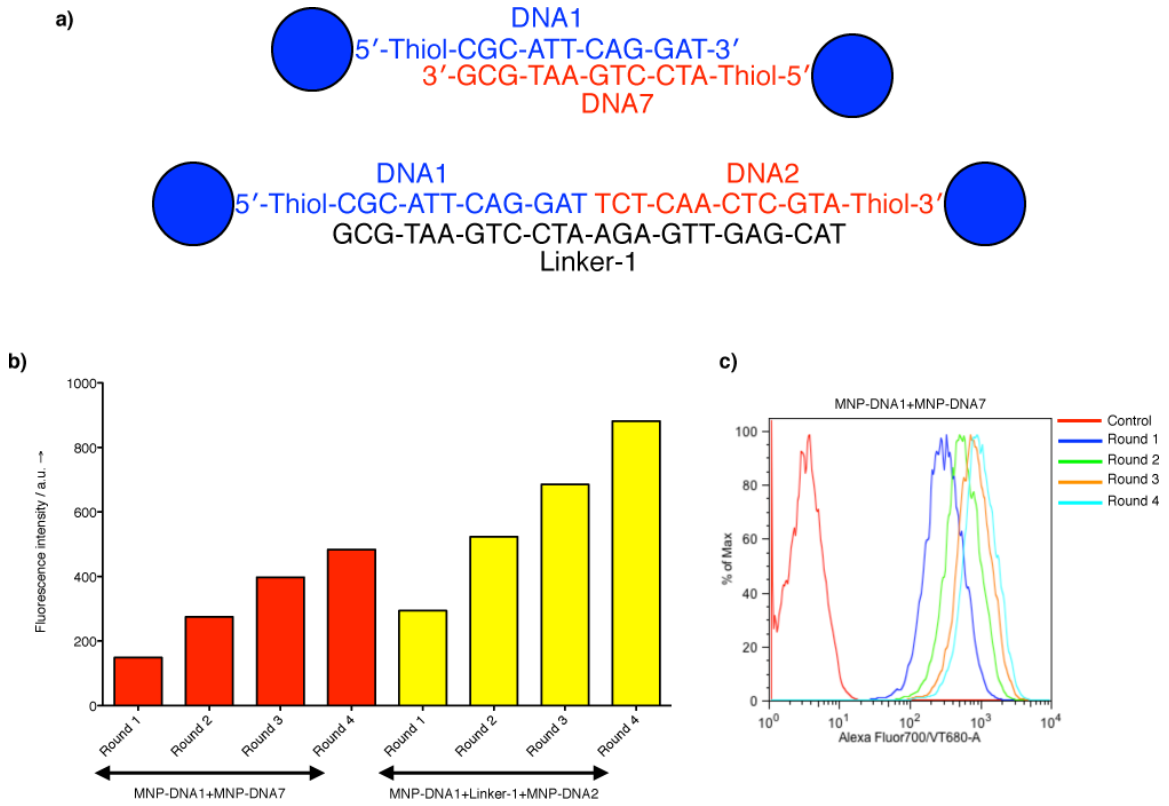


Figure S5. Alternative labeling method for magnetic labeling without *L*-DNA. a) Schematic showing the oligonucleotide sequence for the two different labeling methods. b) Summary of normalized fluorescence intensity. c) A histogram analysis of flow cytometry experiments, comparing the binding efficiency of MNP-DNA probes with and without the use of linker oligonucleotides. For the control samples, mAb-DNA conjugates were omitted during the labeling process.

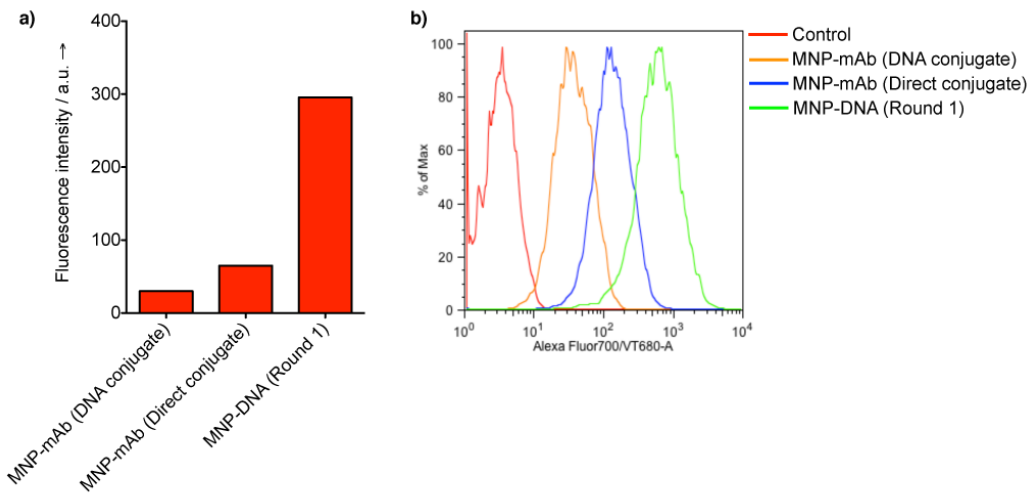


Figure S6. Labeling with direct conjugate probes. The MNP-mAb (direct conjugate) probes were synthesized by attaching reduced anti-HER2 antibodies to maleimide-activated MNPs (see Methods section). The MNP-mAbs (DNA conjugate) were synthesized by mixing HER2-DNA1, Linker-1, and MNP-DNA2 (see Methods section). a) Summary of the normalized fluorescence signal. b) A histogram analysis of flow cytometry experiments comparing HER2/*neu* labeling; use of MNP-mAbs were compared against multi-step labeling with MNP-DNA. The multi-step labeling method using MNP-DNA was more effective than the magnetic labeling method using MNP-mAbs. For the control, samples were incubated with unmodified MNPs.

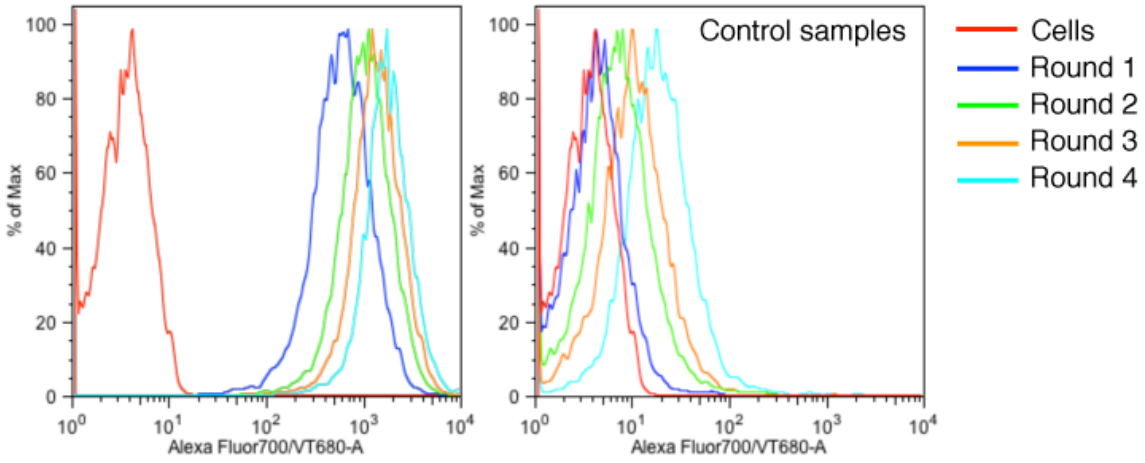
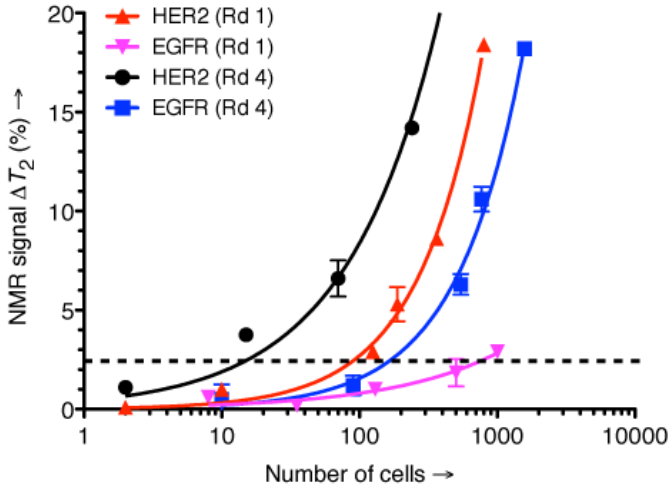


Figure S7. Histogram analysis of flow cytometry experiments for HER2/*neu* magnetic labeling and detection. SK-BR-3 cells were targeted with HER2-DNA1 and labeled with four alternating rounds of VT680-modified MNP-DNA2 and MNP-DNA1 probes. For the control samples, HER2-DNA1 was omitted during the labeling process. The normalized fluorescence signal is summarized in Figure 2c and correlated with μNMR measurements.



Target Marker	Detection Sensitivity
HER2 (Round 1)	150 cells
EGFR (Round 1)	1000 cells
HER2 (Round 4)	20 cells
EGFR (Round 4)	200 cells

Figure S8. µNMR detection of different labeling and targeting methods. The detection sensitivity of high (HER2/*neu*) and low (EGFR) abundance markers, labeled with either a single round or four rounds of MNP-DNA probes, was measured. The detection threshold was determined based on the number of cells required to register a 2.5% change in transverse relaxation time (ΔT_2). Multiple rounds of labeling low abundance biomarkers produced a higher detection sensitivity as well as better diagnosis accuracy.

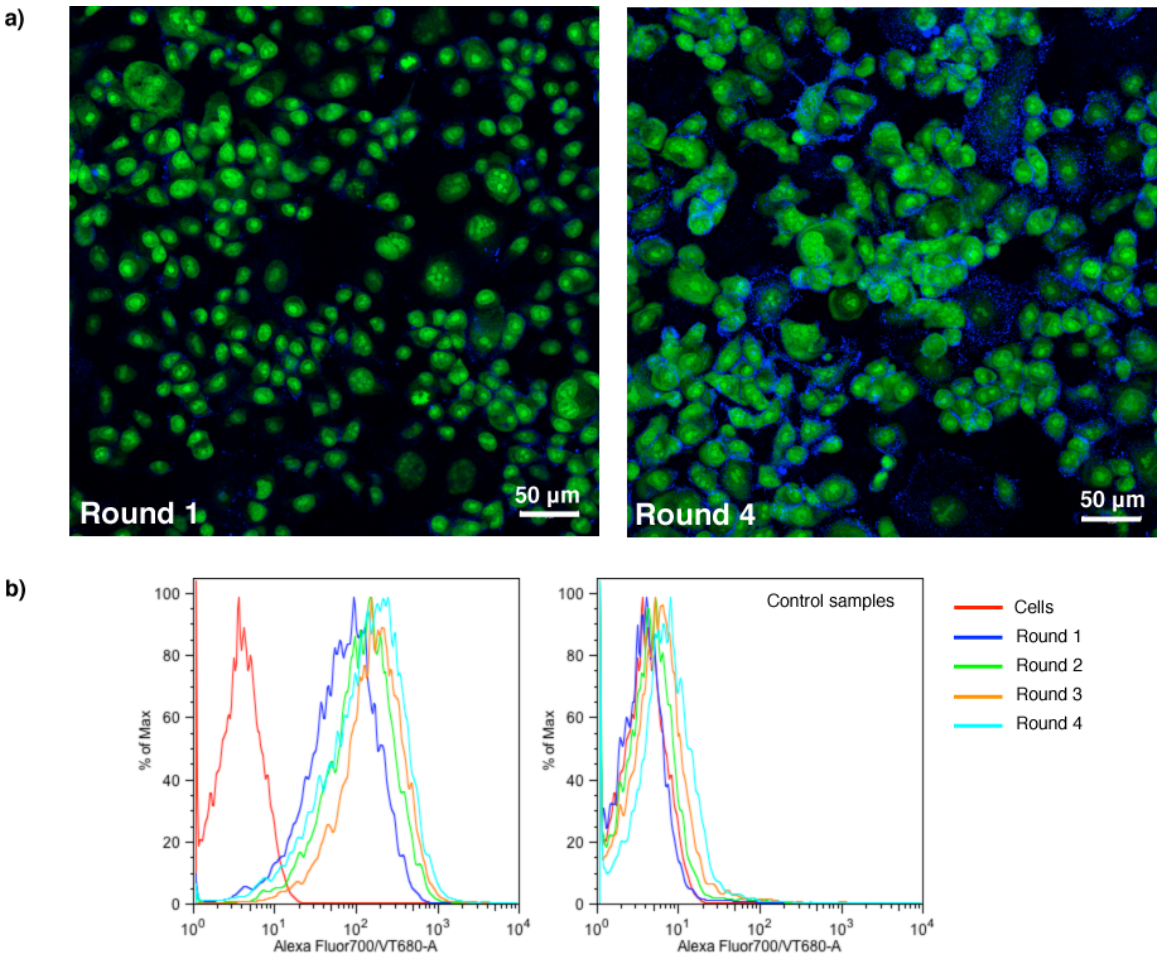


Figure S9. Signal amplification of EGFR markers. SK-BR-3 cells were targeted with anti-EGFR-DNA conjugates and repeated rounds of complementary MNP-DNA. a) Only punctate fluorescence was observed after a single round of MNP-DNA labeling, but strong probe fluorescence was seen after four rounds of labeling. b) A histogram profile of flow cytometry experiments targeting EGFR. For the control samples, anti-EGFR-DNA conjugates were omitted during the labeling process. The normalized fluorescence signal is summarized in Figure 2d and correlated with the µNMR measurements. Green fluorescence: TO-PRO-1 stained nuclei; blue fluorescence: VT680-MNP-DNA.

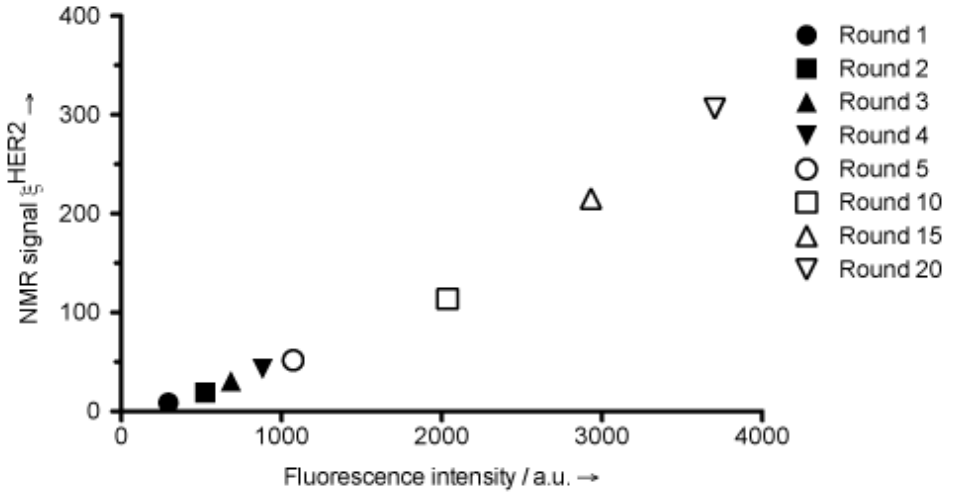


Figure S10. Increased rounds of cell magnetic labeling. Both μNMR and flow cytometry analysis showed increased magnetization and fluorescence with successive rounds of labeling using the MNP-DNA probes.

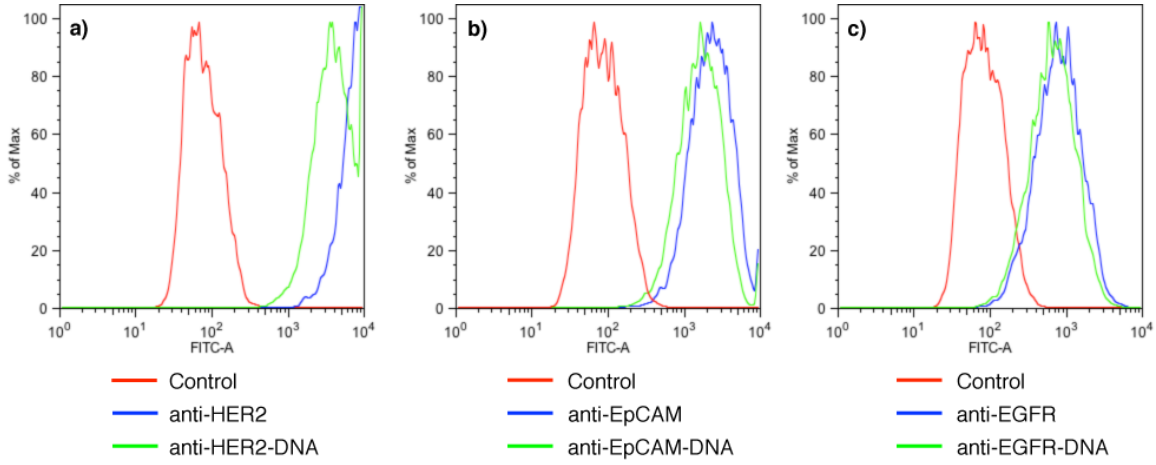


Figure S11. Binding efficiency of antibody-DNA immunoconjugates. SK-BR-3 cells were incubated with either primary antibody or primary antibody-DNA conjugates, before being labeled with secondary antibodies. FITC-labeled Goat anti-Human IgG (Invitrogen) was used as the secondary antibody for both anti-HER2 and anti-EGFR, along with their immunoconjugates. FITC-labeled Rat anti-Mouse IgG 2a/b (BD Biosciences) was used as the secondary antibody for anti-EpCAM, along with its immunoconjugates. Primary antibodies were omitted for control samples and the labeled cells were analyzed using flow cytometry.

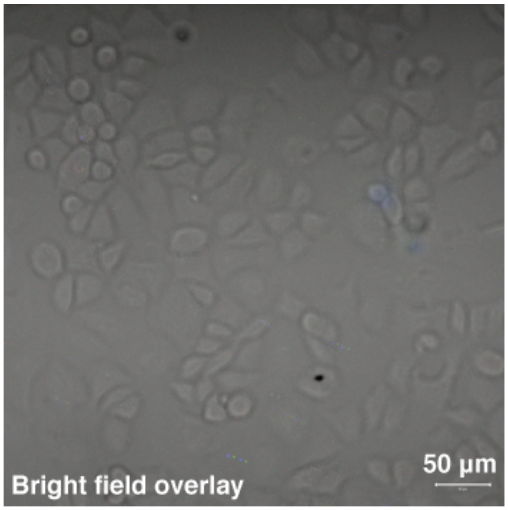


Figure S12. Confocal microscopy analysis. SK-BR-3 control cells were first blocked with all three mAbs (anti-HER2, anti-EpCAM, and anti-EGFR) before incubation with *L*-DNA and MNP-DNAs, similar to the procedure described in Figure 3a. Negligible fluorescence was observed for the control samples.

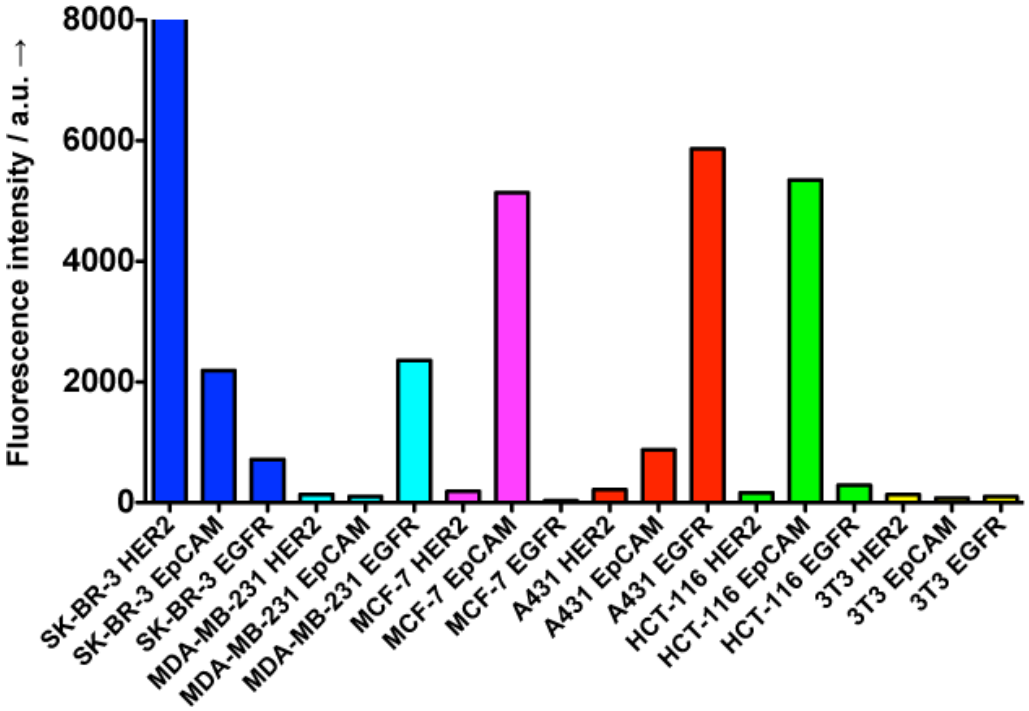


Figure S13. Screening of extracellular markers with secondary antibodies. The five cancer cell lines as well as the fibroblast were incubated with primary antibodies and tagged with fluorescent secondary antibodies. FITC-labeled Goat anti-Human IgG was then used as the secondary antibody to anti-HER2 and anti-EGFR. FITC-labeled Rat anti-Mouse IgG 2a/b was used as the secondary antibody to anti-EpCAM. Primary antibodies were omitted for control

samples and the cells were analyzed using flow cytometry. The normalized fluorescence intensity correlated with the increase in marker expression level.

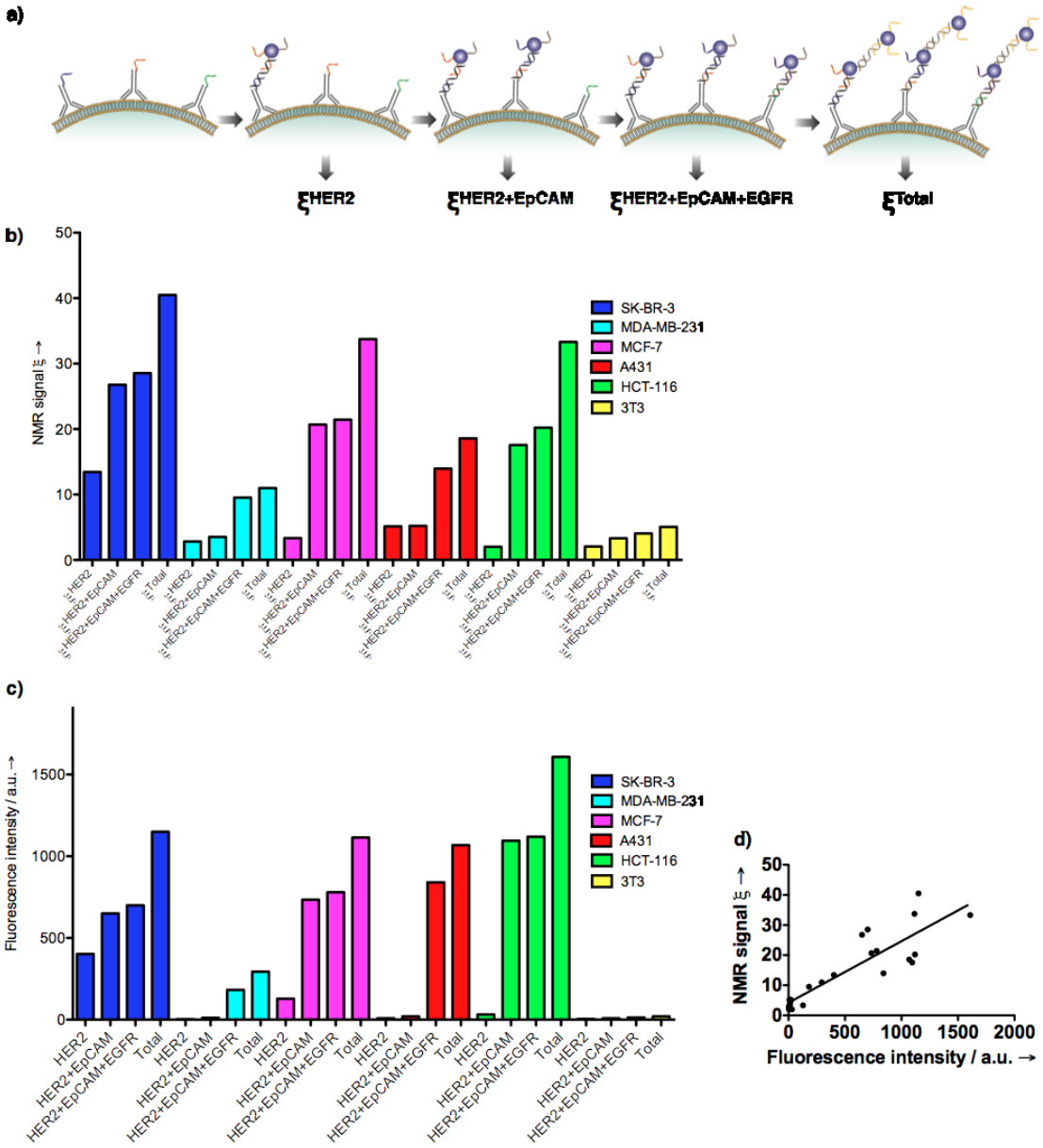


Figure S14. Multiplexed magnetic labeling with µNMR. a) A schematic representation of the multiplexed magnetic labeling described in the Methods section. b) A panel of cancer cell lines as well as the fibroblast control (3T3) were each mixed with all mAb-DNA conjugates. They were then sequentially labeled with MNP-DNA conjugates before being profiled with µNMR. c) Separate flow cytometry experiments were conducted, and d) these were correlated with the µNMR measurements.

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[2] J. B. Haun, C. M. Castro, R. Wang, V. M. Peterson, B. S. Marinelli, H. Lee, R. Weissleder, *Sci. Transl. Med.* **2011**, *3*, 71ra16.