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# Auto-Assembly Protein Arrays for Analyzing Antibody Cross-Reactivity

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# Supporting Information

# Materials and Methods

**Auto-assembly immunoassay protocol**

20 µL of sample was added to the reaction well (with a preprocessed GMR nanosensor array at the bottom of the well with covalently bound capture antibodies) containing a cocktail of the antigen of interest at 10 ng/mL. The following proteins were tested: EGFR, beta 2 microglobulin, survivin, PRDX6, EpCAM, CEA, Serpin, HE4, AGR2, CA125, Trop2, TNF-alpha, Insulin, GCSF, Lactoferrin, VTCN, SLPI, Mesothelin, Eotaxin, and bovine serum albumin (BSA). After 5 minutes incubation, the sample was washed away with a washing buffer (0.1% BSA and 0.05% Tween 20 in phosphate buffered saline, PBS) and 20 µL of magnetic nanoparticle tags, functionalized with streptavidin (MACS 130-048-102, Miltenyi Biotec, Auburn, CA), were added to the reaction well. 10 µL of each biotinylated detection antibody complementary to the target protein of interest at a concentration of 10 µg/mL was introduced sequentially. Upon introduction of each detection antibody, the solution was immediately pipette up and down 10 times over a span of approximately 2 seconds to ensure rapid and thorough mixing in the reaction well. The GMR sensors were monitored in real-time for cross-reactivity of the reagents.

The auto-assembly assay can also be performed completely wash-free. More specifically, after addition of analyte solution to the reaction well, no washing steps are required. For the majority of the experiments presented, washing steps were initially performed in order to minimize the complexity of the reaction taking place so that aberrant binding events can be

indentified with the least ambiguity, however, this assay performs equally well when no wash is performed.

**GMR Sensor Chip Fabrication and Surface Chemistry**

A spin valve film with a layer sequence similar to that of hard disk drives read heads was patterned by ion milling into individual sensors on a 150 µm thermal oxide in a silicon wafer. The GMR spin valve sensor array was fabricated according to Osterfeld *et al.*[12] After fabrication, the GMR sensor surface was first cleaned with acetone, methanol and isopropanol and dried with nitrogen air. Next, a ten minute exposure of the chips to oxygen plasma (PDC-32G Basic Plasma Cleaner, Harrick Plasma) was implemented in order to remove organic materials. Subsequently, a 2% solution of polyethylenimine (PEI, CAS 9002-98-6, Sigma Aldrich) in deionized water was added to the chip. After 3 minutes incubation, the chips were rinsed with deionized water and then baked for 15 minutes at 150 ° C. Next, a piezoelectric non-contact spotter (Scienion sciFlexarrayer from BioDot) was employed to deposit 3 x 360 picoliter droplets of capture antibody (total volume of ~1 nanoleter). Anti-EGFR, anti-CEA, anti-EpCAM, anti-Trop2, anti-beta 2 microglobulin, anti-survivin, anti-PRDX6, anti- serpin, anti-HE4, anti-AGR2, anti-CA125, anti-TNF-alpha, anti-Insulin, anti-GCSF, anti-Lactoferrin, anti-VTCN, anti-SLPI, anti-mesothelin and anti-Eotaxin were each placed over at least three replica sensors at a concentration of 100 µg/mL to 1 mg/mL. In addition, control sensors were covered with 1% BSA in PBS. A group of reference sensors were also deposited with a two component epoxy. The capture probes were incubated for 1 hour at room temperature at 90% relative humidity.

The reaction well was subsequently washed with a washing buffer (0.1% BSA and 0.5% Tween 20 in PBS) and then blocked with 50 µL of 1% BSA in PBS for 30 minutes.

The electronics for reading out GMR sensor arrays were implemented according to Osterfeld *et al.*[12]

**Magnetic label**

The magnetic nanoparticle tags were purchased commercially from Miltenyi Biotech Inc. The magnetic nanotags are comprised of about a dozen 10 nm iron oxide nanoparticles embedded in a dextran polymer with ~10% magnetic material (wt./wt.). The surface of the magnetic nanotag is functionalized with streptavidin. The entire nanoparticle is approximately 50 nm in diameter, the zeta potential is -11 mV, and the translational diffusion coefficient is $8.56 \times 10^{-12}$ $m^2 s^{-1}$. The particles are colloidally stable so they do not settle non-specifically on the sensor surface.

# Supplementary Figures and Text

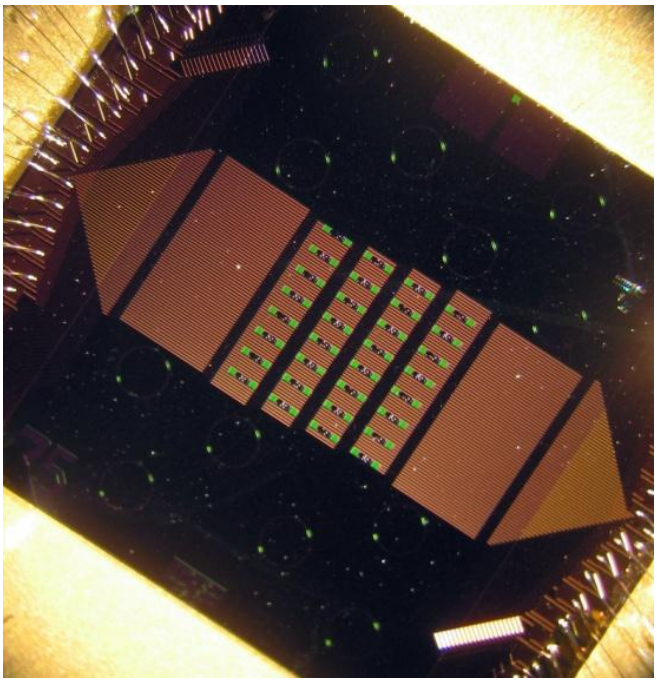

**Figure S1| GMR nanosensor array**

Photograph of the array of GMR nanosensors. Each green square in the image represents one nanosensor in an 8x8 array. The GMR nanosensors are built with an ultrathin passivation making them well suited for the novel auto-assembly assay. The GMR sensors are proximity based sensors, therefore, only magnetic nanotags within ~150 nm of the surface are detected(12). Since the magnetic nanotags are only 50 nm in diameter and colloidally stable, they do not settle or precipitate, contributing negligible signal in the absence of the target protein or detection antibody. Only in the presence of both the biomolecule of interest and detection antibody will the magnetic nanotags congregate over the appropriate sensor in close enough proximity and in high enough concentration for the GMR sensor to experience a measurable MR change. This is a significant advantage over the vast majority of protein detection platforms in which the excess/unreacted nanotags must be washed away prior to detection, limiting their ability to monitor antibody binding events over time. Similarly, the surface chemistry of the magnetic sensor array exhibits negligible non-specific binding. As a result, while performing a traditional sandwich assay requires washing steps to remove excess antibodies or nanotags, with this new technology, the signal remains unchanged prior to and after a final washing step. Accordingly, it

is equally valid to determine the protein content in the assay prior to implementing a final wash (**Fig. S6**). Thus, the assay obviates the need for washing steps, and offers a faster, simpler testing process that untrained users can easily perform.

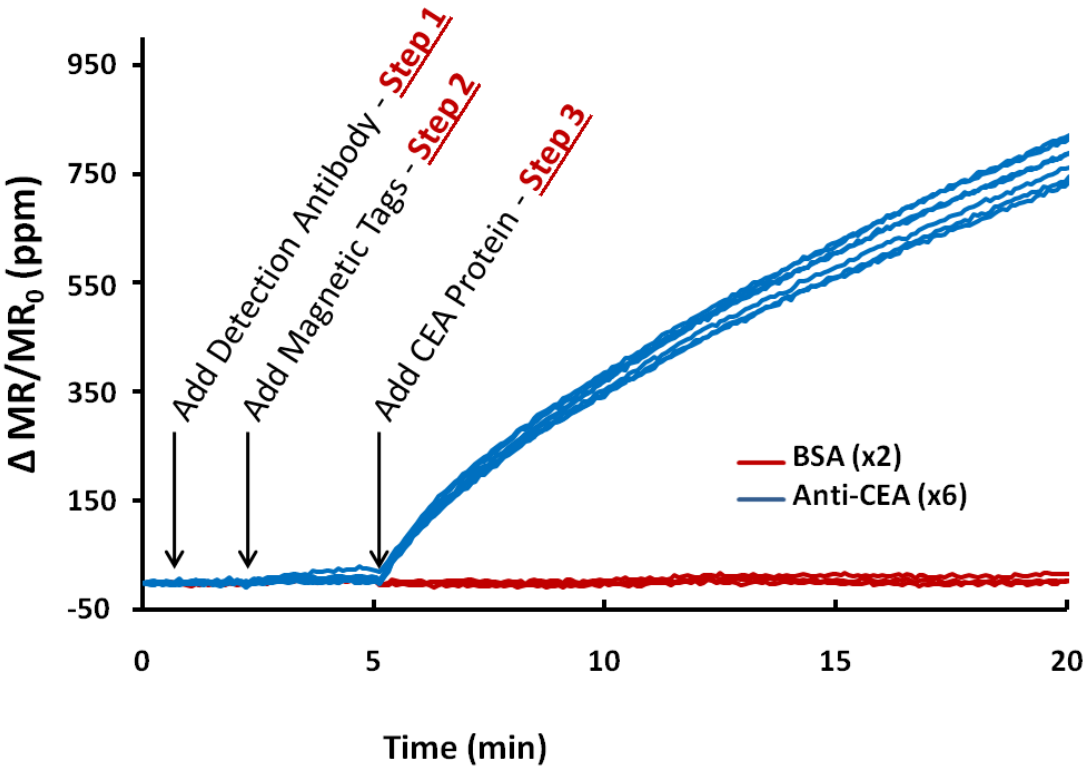


**Figure S2| Real-time binding auto-assembly assay for antigen detection**

The auto-assembly immunoassay discussed can also be utilized to monitor antigen binding to capture antibody by reorganizing the steps in the assay. Addition of biotinylated anti-CEA detection antibody followed by magnetic nanotags contribute negligible signal, however, once the protein of interest is introduced, the magnetic nanotags are detected by the sensors with the appropriate capture antibody. The negative control sensors, coated with BSA, remain flat, indicating negligible cross-reactivity. The y-axis units are the change in magnetoresistance normalized to the initial magnetoresistance presented in parts per million (ppm).

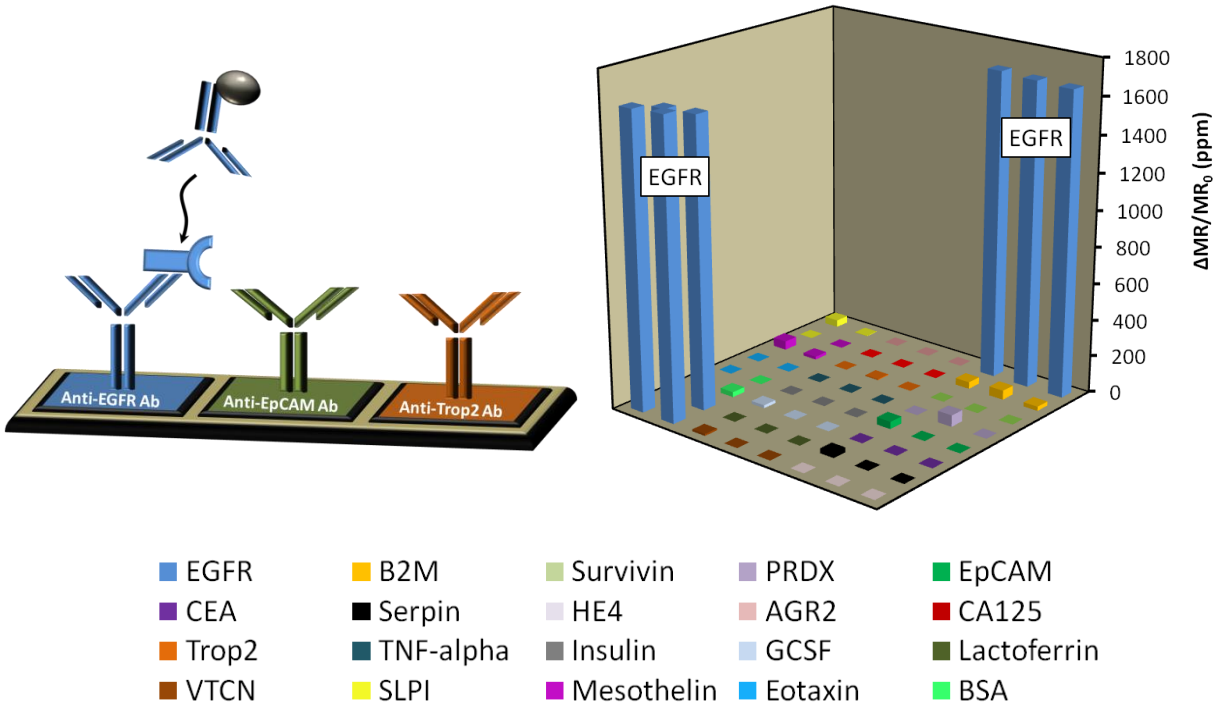


**Figure S3| EGFR control Experiment**

Control experiment where EpCAM protein and Trop2 protein were intentionally excluded from the reaction well, all other proteins were included. Upon addition of anti-EGFR detection antibody, only the EGFR sensors gave detectable signal indicating that the anti-EGFR detection antibody does not bind to any of the capture antibodies employed, the anti-EGFR detection antibody only aberrantly binds to the Trop2 and EpCAM proteins themselves when they were included (**Fig. 3** in the main text).

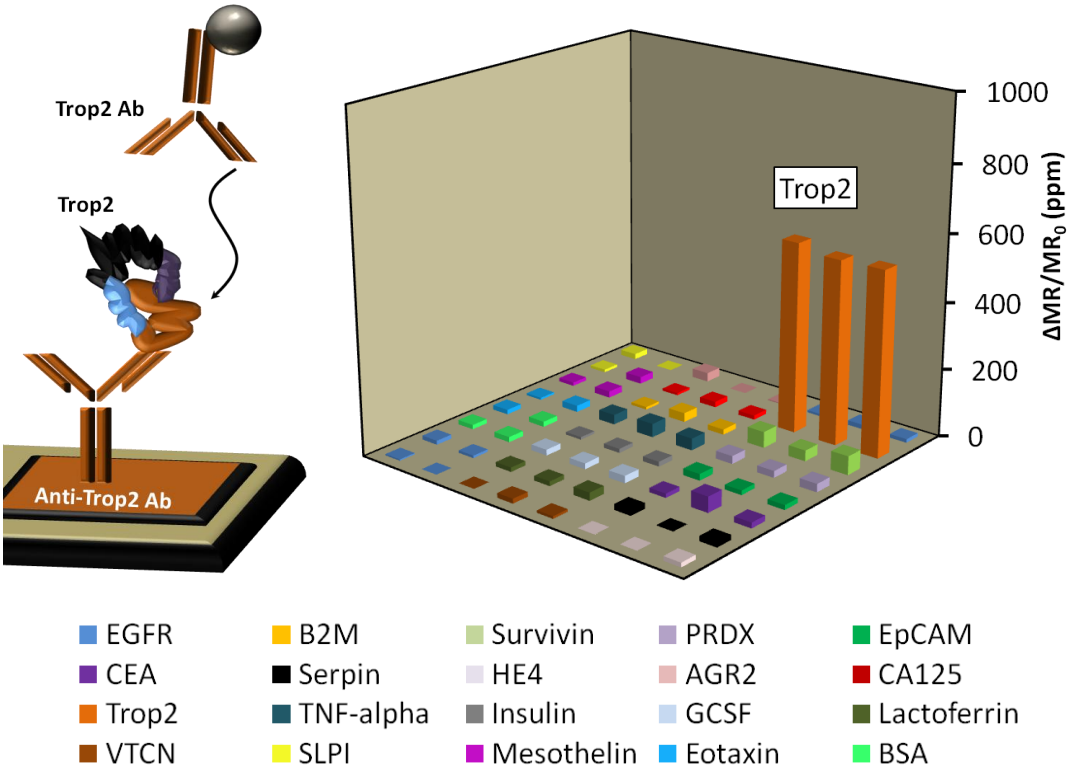


**Figure S4| Trop2 Control Experiment**

Control experiment where EGFR protein was intentionally excluded from the reaction well, all other proteins were included. Upon addition of anti-Trop2 detection antibody, only the Trop2 sensors gave detectable signal indicating that the Anti-Trop2 detection antibody does not bind to any of the protein and capture antibodies employed.

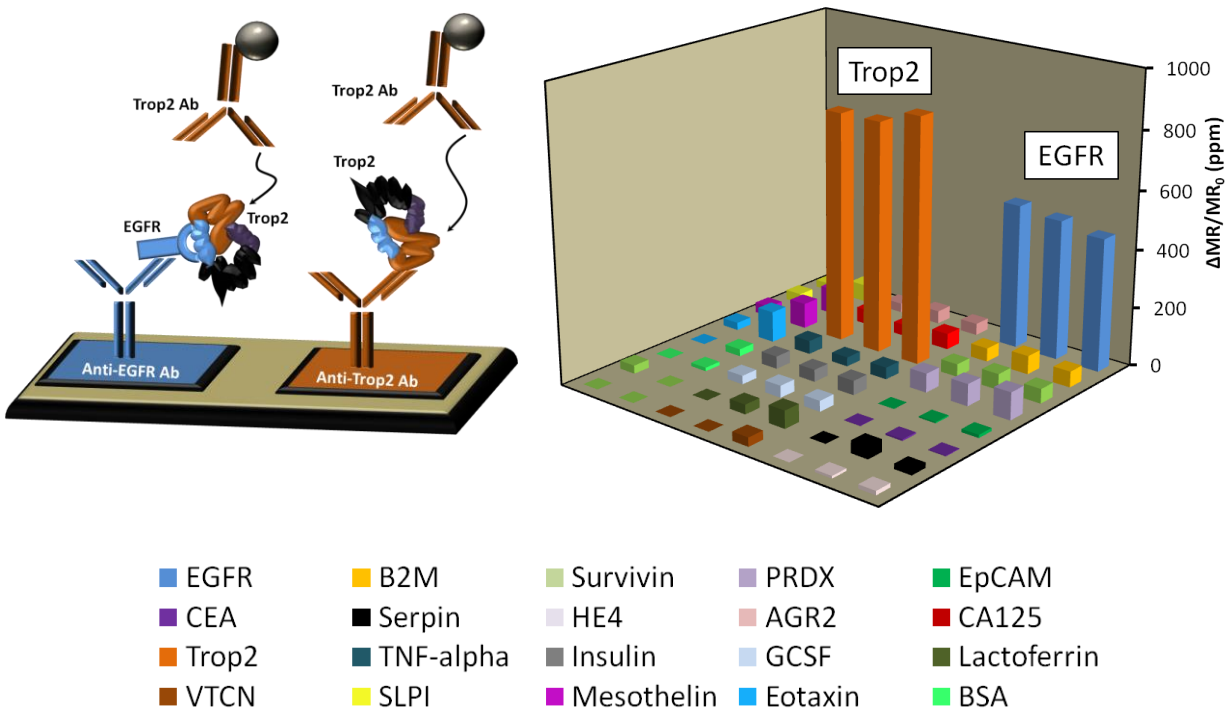


**Figure S5| Auto-assembly immunoassay investigating anti-Trop2 detection antibody cross-reactivity**

20 unique capture antibodies were selectively immobilized on unique sensors in replicas of 3-7. All 20 proteins were incubated at high concentration (approximately 10 ng/mL). After washing, magnetic nanotags were added followed by anti-Trop2 detection antibody. The auto-assembly assay was run for 5 minutes resulting in signal over the EGFR sensors and Trop2 sensors. The data shows that Trop2 protein can bind to EGFR protein, facilitating aberrant binding of Trop2 detection antibody which results in nonspecific signal at the EGFR sensor.

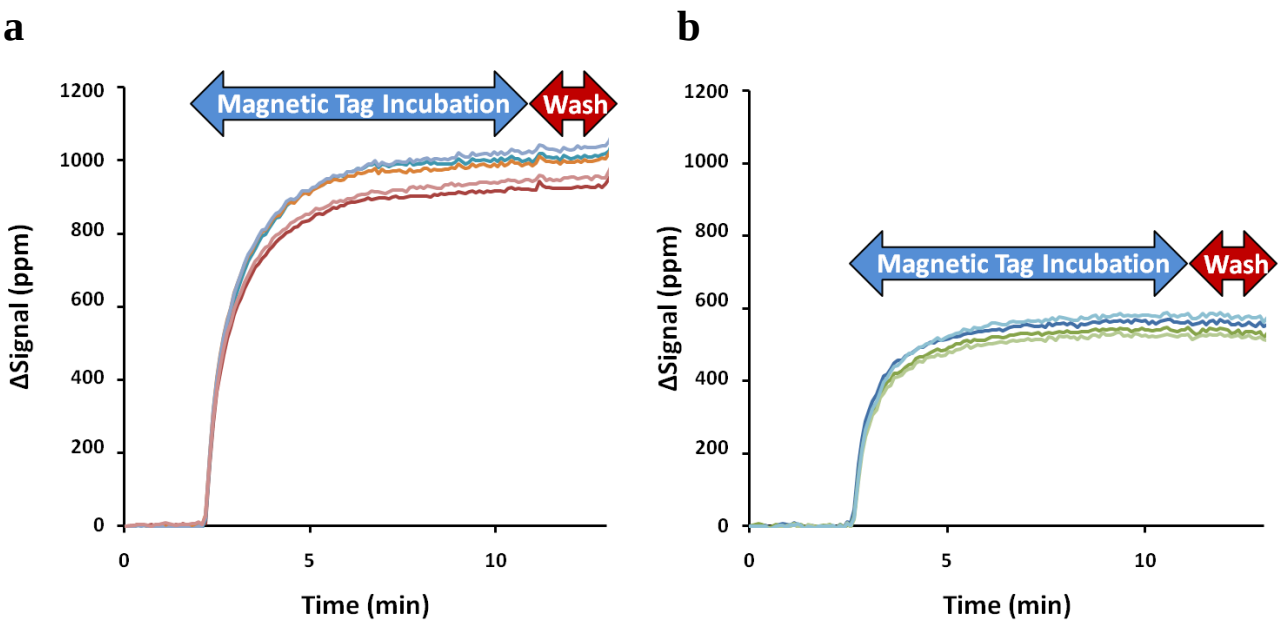


**Figure S6| Minimal non-specific binding.**

Shown above are binding curves for carcinoembryonic antigen (CEA) using a traditional sandwich assay. In this format, the final washing step (of three in total) is used to remove the unbound magnetic nanotags. However, as a direct result of the minimal non-specific binding (shown via BSA control sensor in **Fig. S2**), the signal remains unchanged after the final washing step, permitting removal of the final washing step without distorting the final signal.

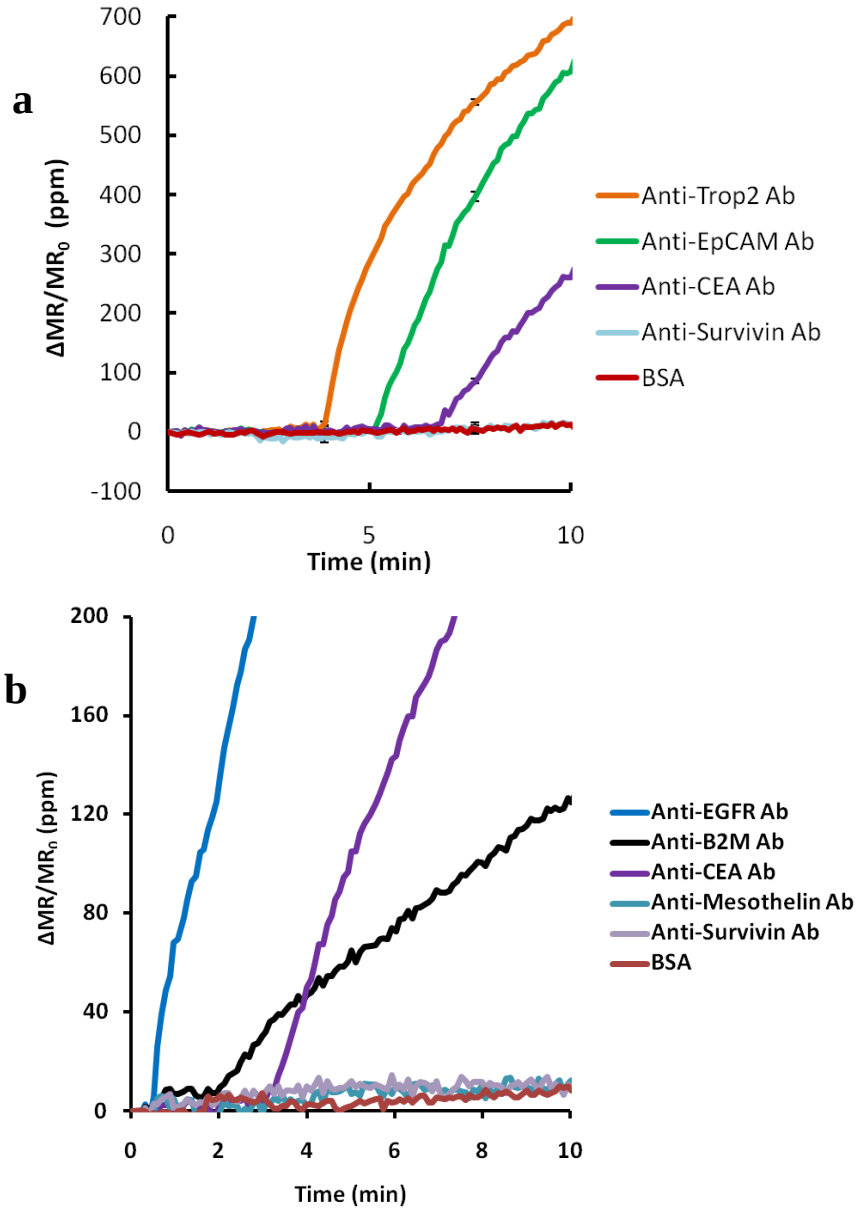


**Figure S7| Control experiments without cross-reactivity**

(a) Introduction of Anti-Trop2 detection antibody, Anti-EpCAM detection antibody and Anti-CEA detection antibody reveal no cross-reaction or non-specific binding among the proteins tested, when cross-reactive reagents are excluded. (b) Similarly, in a separate set of experiments, introduction of anti-EGFR antibody, anti-B2M antibody and anti-CEA antibody reveal no cross-reaction or non-specific binding among the proteins tested. In addition, it is apparent that the anti-EGFR antibody and anti-CEA antibody have higher binding affinities than anti-B2M antibody as noted by the more rapid rise in signal upon addition of the detection antibody.