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Säll, Anna; Carlsson, Fredrika; Olsson, Niclas; Wingren, Christer; Ohlin, Mats; Persson, Helena; Waldemarson, Sofia

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# AFFIRM – a multiplexed immunoaffinity platform that combines recombinant antibody fragments and LC-SRM analysis

*Anna Säll<sup>‡</sup>, Fredrika Carlsson<sup>‡1</sup>, Niclas Olsson<sup>2</sup>, Christer Wingren, Mats Ohlin,*

*Helena Persson<sup>†</sup>, Sofia Waldemarson<sup>†\*</sup>*

Department of Immunotechnology, Lund University, Medicon Village (House 406), 223 81  
Lund, Sweden

*E-mail addresses:* Anna.Sall@immun.lth.se (Anna Säll)  
fredrika@scripps.edu (Fredrika Carlsson)  
nolsson@stanford.edu (Niclas Olsson)  
Christer.Wingren@immun.lth.se (Christer Wingren)  
Mats.Ohlin@immun.lth.se (Mats Ohlin)  
Helena.Persson@immun.lth.se (Helena Persson)  
Sofia.Waldemarson@immun.lth.se (Sofia Waldemarson)

## KEYWORDS

Affinity proteomics, SRM, scFv, serum, biomarker

## ABSTRACT

Targeted measurements of low abundance proteins in complex mixtures are in high demand in many areas, not the least in clinical applications measuring biomarkers. We here present the novel platform AFFIRM (AFFInity sRM) that utilizes the power of antibody fragments (scFv) to efficiently enrich for target proteins from a complex background and the exquisite specificity of SRM-MS based detection. To demonstrate the ability of AFFIRM, three target proteins of interest were measured in a serum background in single- and multiplexed experiments in a concentration range of 5-1000 ng/ml. Linear responses were demonstrated down to low ng/ml concentrations with high reproducibility. The platform allows for high throughput measurements in 96-well format and all steps are amendable to automation and scale-up. We believe the use of recombinant antibody technology in combination with SRM MS analysis provides a powerful way to reach sensitivity, specificity and reproducibility as well as the opportunity to build resources for fast on-demand implementation of novel assays.

INTRODUCTION

Quantitative measurements of specific target proteins with high precision and quantitative accuracy have a wide range of application areas, not the least in clinical research and analysis. Proteins carrying clinical information, referred to as biomarkers, hold the promise to revolutionize medicine if clinically implemented. Developing assays for measuring these however still remains challenging <sup>1</sup>. Biomarkers need to be assessed in large sample cohorts with high accuracy and reproducibility, and combining several biomarkers can potentially increase the precision. For true clinical utility, these proteins should also preferably be measurable in an easily accessible body fluid such as blood for a minimally invasive test. This poses further challenges as serum or plasma samples represent proteins in a dynamic range of over 10 orders of magnitude <sup>2</sup>. Hypothesizing that the target proteins are produced at the site of disease and shed into the blood stream, the enormous dilution effect makes them likely to be present in the low ng/ml concentration or below <sup>2,3</sup>. These requirements all place enormous demands on technology to deliver accurate and reproducible data yet with relatively high throughput.

Immunoassays, often sandwich enzyme-linked immunosorbent assays (ELISA), are today considered golden standard for quantitative clinical measurements of proteins in solution <sup>4,5</sup>. Despite their extensive utility, these assays suffer from certain shortcomings. High-quality ELISAs normally require pairs of antibodies <sup>6</sup>. However, development of appropriate antibody pairs having high enough sensitivity and specificity is a demanding task. Multiplexing ELISAs also remains challenging <sup>7</sup>. In addition, many disease-related proteins are post-translationally modified, truncated or are present in active or inactive forms depending on sequence variations. These variants might be the ultimate discriminatory biomarkers <sup>8-10</sup>. However, most antibodies are unable to discriminate between such small sequence deviations.

Mass spectrometry (MS) based technologies have therefore been proposed as an attractive alternative to ELISA. In particular, for more quantitatively accurate measurements selected reaction monitoring (SRM) mass spectrometry, also known as multiple reaction monitoring (MRM), has increasingly been applied to measure predetermined sets of proteins. SRM has high multiplexing capability and can measure up to hundreds of proteins in one single analysis, is highly specific through its sequence based readout and has been demonstrated to provide reproducible measurements<sup>11,12</sup>. This technology has routinely been used in the clinic for years for high-throughput quantification of metabolites in plasma<sup>13</sup>. SRM has more recently been demonstrated to also provide measurements of peptides in plasma with the precision and reproducibility and the transferability between laboratories required for clinical utility<sup>12,14-16</sup> and can provide highly sensitive measurements in the low ng/ml range<sup>17,18</sup>. This, however, requires extensive sample workup and peptide separation, procedures that are hardly amenable to high-throughput analysis. Therefore targeted enrichment has often been combined with SRM readout<sup>19</sup>.

Antibodies possess the unique property of selectively binding proteins with high specificity and sensitivity, and they can thus be used to increase the selectivity and sensitivity of an assay. The attractive prospect of combining the sensitivity of antibody enrichment with the specificity of mass spectrometry has fueled the development of platforms for targeted analyses with the aim of providing robustness and throughput for quantitative protein measurements<sup>19-23</sup>.

Here we present a novel developed platform, AFFIRM (AFFinity sRM), that combines protein enrichment by specific recombinant antibody fragments, in form of single chain variable fragments (scFvs), generated in house by phage display technology<sup>24,25</sup>, with liquid chromatography (LC)-SRM MS readout. An scFv is a fusion protein of the variable domains of the heavy and light chains of an antibody that are connected by a short peptide linker.

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Despite the removal of the constant domains, this protein retains the specificity of the original antibody. Such recombinant antibody fragments represent a sustainable resource that allows fast production and is amenable to high-throughput generation of specific binders<sup>26-29</sup>. The AFFIRM platform has been developed for a high-throughput workflow in a 96-well format using a magnetic bead processor for complete automation of capture and wash steps (Figure 1). In this proof-of-concept study, six recombinant scFvs were developed against three target proteins. Each scFv was coupled to magnetic beads and used to capture the corresponding target protein from a serum background in both a single- and multiplexed fashion. The captured proteins were digested directly on the beads followed by direct LC-SRM peptide analysis for protein quantification, providing a completely automated protocol performed within one day. By making use of recombinant antibody fragments for enrichment of target proteins in combination with quantification through SRM, this integrated platform promises to be an attractive alternative for quantitative measurements of clinical biomarkers in complex backgrounds such as plasma or serum.



## EXPERIMENTAL PROCEDURES

*Proteins* - Three target proteins, human keratin-19 (KRT19) (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel), the C-terminal located BRCT domain of human breast cancer type 1 susceptibility protein (BRCA1) (kindly provided by Michael Sandrini, University of Copenhagen, Denmark) and the extra-cellular domain of 32 tandem repeats of human mucin-1 (MUC1) fused to a mouse IgG<sub>2a</sub> Fc region (MUC-Fc)<sup>30</sup>, kindly provided by Gunnar C. Hansson (Gothenburg University, Sweden) were used in this study.

*Synthetic antibody libraries* – Two different human synthetic scFv libraries were used for phage selections on the three target proteins. For selections on KRT19 the in-house designed and constructed HELL-11 library (Säll and Persson *et al*, manuscript in preparation), was used. In short, built on the framework of immunoglobulin genes IGHV3-23 and IGK1-39, diversity was added into defined codons in four of the six complementarity determining regions (CDR); CDR-H1, CDR-H2, CDR-H3 and CDR-L3, using a tailored oligonucleotide mixture together with an optimized Kunkel mutagenesis methodology as previously explained<sup>58, 59</sup>. The procedure yielded a highly diverse repertoire containing  $>1 \times 10^{10}$  unique members, as measured by the number of bacterial colonies obtained after transformation. For selections on BRCA1 and MUC1-Fc an antibody library synthesized by GENEART AG (Regensburg, Germany) was used. Based on a crystal structure of a MUC1 specific scFv (Wingren *et al.*, unpublished), a total fourteen surface-exposed residues, were diversified in the three CDRs of the heavy chain (IGHV3-23), and the third CDR of the light chain (IGLV1-47), giving a theoretical diversity of  $1 \times 10^{11}$ . In practice, a library size of  $1 \times 10^{10}$  was obtained. The bacteria harboring the libraries were infected with M13K07 helper phages (New England Biolabs,

Ipswich, MA, USA) and scFv displaying phages were harvested by standard polyethylene glycol/NaCl precipitation.

*Selection of specific binders using phage display* – Phage display selections were performed on KRT19, BRCA1 and MUC1-Fc using two different selection procedures. In short, the antigens (KRT19 and BRCA1) were either immobilized in immunotubes (Nunc A/S, Roskilde, Denmark) or coupled (MUC1-Fc) to magnetic Pan Mouse IgG beads Dynabeads (Dynal A/S, Oslo, Norway). Prior to the selection, the phage stocks were incubated with streptavidin immobilized in immunotubes or against Pan Mouse IgG beads but with no antigen for 1 hour at room temperature and slow rotation. Subsequently, the phage stock was transferred to the antigen-coated immunotubes or to the antigen coated magnetic beads and incubated for 2 h at room temperature and slow rotation. The immunotubes and magnetic beads were washed 6-11 times. Bound phages were eluted by trypsin, which cleaves the trypsin-sensitive site located between the displayed scFv and gene III, amplified in solution over night at 30 °C, PEG-precipitated, resuspended in PBS containing 0.1-0.5% BSA (w/v) and used for subsequent rounds of selection as described above. In total, four rounds of selection were carried out for KRT19, three rounds of selection for BRCA1 and two rounds of selection for MUC1-Fc. For each selection round the amount of antigen was gradually decreased and the number of washes increased.

*Production and purification of soluble antibody fragments* – Vectors encoding scFv specific for KRT19 and BRCA1 were digested with *Sfi*I and *Avr*II and subsequently re-ligated into a vector that allows for production and purification of soluble scFv. In order to remove gene 3 from the phagemids encoding the MUC1-Fc specific scFv, these were digested with *Eag*I and re-ligated. Both these procedures provide the secreted scFv with a hexahistidine tag at the C-

terminus, preceded by either a trippel-FLAG tag or a single FLAG tag. The constructs were transformed into Top10 *E.coli* and soluble scFv were produced and purified essentially as described<sup>31</sup>. The integrity and purity of the scFv was confirmed by SDS-PAGE.

*Immunoassay to determine specific binding of selected scFv* – In order to evaluate the specificity of the generated scFv, individual phage-displayed scFv clones or their soluble counterparts were screened for binding specificity in ELISA. 96- or 384-well plates (Corning Inc., New York, NY, USA) were immobilized with recombinant KRT19, BRCA1, MUC1 or with BSA and incubated overnight. Unbound proteins were removed by washing and the wells were blocked with BSA. Phages displaying scFv or soluble scFv were allowed to bind and after another round of washing HRP-labeled mouse anti-M13 monoclonal antibody (GE Healthcare, Buckinghamshire, UK), HRP-labeled mouse anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO, USA) or HRP-labeled mouse anti-His<sub>6</sub> antibody (Roche Applied Science, Sweden) were applied to determine the level of bound phages or scFv. Ultra TMB-ELISA (Thermo Scientific Pierce, Rockford, IL, USA) was used as chromogen. The reaction was stopped by the addition of 2 M sulfuric acid and the absorbance was measured at 450 nm.

*Selection of target peptides and transitions* – Peptides and transitions relevant for analysis of KRT19, BRCA1 and MUC1-Fc were selected in the SRM management software Skyline<sup>32</sup>. An *in-silico* trypsin digestion of the target proteins allowing peptides of 7-25 amino acids length was performed and peptides with C, M, H, NXT/NXS or RP/KP in the sequence were removed. The peptides were checked against a background proteome of the entire human proteome (UniProt KB download) to ensure that truly unique proteotypic peptides were selected for each protein. MS/MS data of digested recombinant target proteins was obtained on a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Electron, Bremen, Germany)

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coupled to an Eksigent 2D NanoLC system (Eksigent technologies, Dublin, CA, USA), equipped and run as described below for LC-SRM. This data was used to select peptide charge-state and their 4-5 best transitions. The final number of target peptides was narrowed down to three peptides for KRT19 and two for BRCA1 plus two mouse IgG peptides that were used for measuring the MUC1-Fc fusion protein (Table 1). In addition to the peptides representing the target proteins, peptides for three proteins (apolipoprotein B-100, Immunoglobulin gamma-1 chain C region and Complement C3) were identified. These proteins are highly abundant in serum and they were identified in the captured material likely due to non-specific binding. These targets were included in the SRM assay represented by one peptide each. Two peptides representing clones specific for either KRT19 or BRCA1/MUC-Fc were also included, giving a final SRM assay consisting of 12 peptides and 81 transitions. Collision energies were optimized through Skyline using trypsin digests of the recombinant proteins. Protein names, peptide sequences, transitions and collision energies are given in Supplementary Table S1.

*Capture of target proteins in a serum background* – Purified soluble scFv were coupled to magnetic Dynabeads M-270 Epoxy beads (Invitrogen Dynal AS, Oslo, Norway) according to manufacturer’s protocol. Briefly, coupling was performed by mixing 0.9 mg beads with 0.1 M sodium phosphatase (pH 7.4), 20 µg scFv and 1 M ammonium sulfate. The mixture was incubated over night. The two scFvs developed against the same target protein were mixed before coupling. Capture of spiked proteins in serum was carried out using a KingFisher magnetic bead processor (Thermo Scientific, Waltham, MA, USA). The automated steps included two washes of the 20 µl scFv-coupled beads used for each capture, in 1 PBS containing ml 0.05% (v/v) Tween20. Following the washes, the beads were incubated in 10% (v/v) human serum (Sigma-Aldrich Corp, St. Louis, MO USA) diluted in PBS and spiked

with target proteins at a concentration of 0, 5, 25, 50, 100, 500, 1000 ng/ml for 1 h. The beads were washed 4 times in PBS containing 0.03% CHAPS (w/v) and once in 50 mM ammonium bicarbonate containing 0.03% (w/v) CHAPS and subsequently transferred to Axygen maximum recovery tubes. The wash solution was removed prior to addition of 15  $\mu$ l 12 ng/ml trypsin (sequencing grade modified, Promega Corp, Madison, WI, USA). Trypsin digestion was performed at 37°C for 4 h at which time another 5  $\mu$ l trypsin was added and the digestion was continued for an additional 1 h. 2  $\mu$ l 5% (v/v) formic acid (FA) was added, the samples were dried and reconstituted in 15  $\mu$ l 0.1% (v/v) FA, 3% (v/v) acetonitrile (ACN) and transferred to HPLC vials for LC-SRM. For multiplexed captures 20  $\mu$ l scFv-coupled beads per target protein were mixed and used for capture. 30  $\mu$ l 12 ng/ml trypsin was used followed by another 5  $\mu$ l addition after 4 h, the samples were dried and reconstituted in 15  $\mu$ l 0.1% FA, 3% ACN. 6  $\mu$ l sample was injected for LC-SRM analysis.

*Serum dilution curves* – 10% serum was spiked with the three target proteins at a concentration range of 5-100 000 ng/ml (5, 25, 50, 100, 500, 780, 1560, 3125, 6250, 12500, 50000, 100000 ng/ml). Captures were also performed from serum with no spiked proteins to provide blank measurements. The samples were digested. Briefly, dried samples were reconstituted in 6M urea, 100 mM Tris, pH 8 and incubated at 37°C for 30 min. The samples were diluted 10x in 100 mM Tris, pH 8 and trypsin was added at a trypsin/sample ratio of 1:50. The sample were digested over night (16h) and 10% (v/v) FA was added to a pH of 3. The samples were cleaned using C18 UltraMicroSpin Columns, 3-30  $\mu$ g capacity (The Nest Group). The peptides were dried and reconstituted in 0.1% FA, 3% ACN and transferred to HPLC vials for LC-SRM using the same setup as for the AFFIRM assay. An equivalent of 1  $\mu$ g total protein was injected.

*Nano-LC-SRM* – An Eksigent 2D NanoLC system (Eksigent technologies, Dublin, CA, USA) connected to a Triple Stage Quadrupole Mass Spectrometer (TSQ Vantage, San José, CA) was used for LC-SRM analysis. Solvents used were water/0.1% FA (v/v) (mobile phase A) and ACN/0.1% (v/v) FA (mobile phase B). The LC system was equipped with a trapping column (0.3 mm id, 5 mm, PepMap Acclaim C18, LCPackings, Sunnyvale, CA). Analytical columns were packed in-house in 0.075 mm id, 100 mm PicoFrits (New Objective, Woburn, MA) with 3 µm ReproSil C18-AQ particles (Dr. Maisch, Germany) to a length of 12 cm. The LC gradient was delivered at 300 nl/min and consisted of a linear gradient of mobile phase B developed from 3–35% B during 22 min and from 45-90% during 8 min. An injection for wash and equilibration of the column was performed between each sample. The Triple Stage Quadrupole Mass Spectrometer (TSQ Vantage, San José, CA) was equipped with a nano electrospray interface operated in the positive ion mode with a spray voltage of 1.8 kV and an ion capillary temperature of 270 °C. Both Q1 and Q3 were set to unit resolution (0.7 Da) and 10 ms dwell time was used. Data were acquired using the Xcalibur software (version 2.0.7).

*Data Analysis* – Raw files from SRM data acquired on the TSQ Vantage were analyzed and manually inspected in Skyline. Peak areas for the measured SRM transitions for each individual peptide were integrated and summed to generate the peptide peak areas. Data was exported from Skyline as .csv files and response curves for the different proteins' spike levels were plotted to monitor the linearity of the response.

## RESULTS

We present here the immunoaffinity SRM platform AFFIRM developed for quantification of low-abundance proteins of interest in complex samples such as serum. This is done in an assay format where target proteins are enriched using human recombinant scFv coupled to magnetic beads with subsequent on-bead trypsin digestion and LC-SRM readout. As part of the AFFIRM platform, we continuously develop in-house scFv and SRM assays against target proteins of interest (Figure 1A). Once developed, these resources are used for building an assay that can quickly be established in a plug-and-play fashion with minimum preparative steps (Figure 1B). The AFFIRM assay is automated and optimized for minimum sample handling and high-throughput (Figure 1C). Three target proteins, KRT19, BRCA1 and MUC1-Fc (Table 1), were used to demonstrate the proof-of-concept of this novel platform.

*Selection and screening of protein specific scFv* – Human recombinant antibody fragments against KRT19, BRCA1 and MUC1-Fc were generated from in-house designed antibody libraries using phage display technology. Based on performance in an initial phage ELISA analysis, two scFvs specific for each antigen were chosen for sub-cloning and evaluation for the intended application. ELISA of the soluble expressed scFv verified specific recognition of the three targets (Figure 2). ScFv specific for the MUC1-Fc fusion protein were shown in separate experiments to recognize synthetic peptides covering the MUC-1 repeat sequence in the absence of the Fc (data not shown). Sequence analysis of the specific clones further verified uniqueness of the six clones (data not shown).

*SRM analysis* – Each target protein was measured through 2-3 proteotypic peptides with 4-5 transitions each (Table 1). In addition, one peptide from each of the three serum proteins, Apolipoprotein B-100, Immunoglobulin G1 and Complement C3 that gave the highest

background signals in the orbitrap experiments were included (data not shown), as well as two peptide representing the scFvs.

*The AFFIRM assay workflow* – Enrichment of recombinant proteins from human serum samples were performed in an automatic KingFisher bead processor. The 90-minute processing included automatic washing of antibody fragments coupled to magnetic beads, a 1-h binding cycle, followed by five wash steps to minimize background signal. The beads were transferred to the Axygen tubes and the captured proteins were digested directly on the beads with trypsin for 5 h. This digestion protocol was the final choice of several tested, including elution of target protein followed by digestion with or without reduction and alkylation (data not shown). In addition to being the fastest protocol, the elution step before trypsin digestion can be omitted and, thus, variations due to incomplete elution, pipetting and protein adhesion to the tubes are avoided<sup>33</sup>. Five hours of trypsin digestion also gave superior results compared to over night digestion, probably due to the low sample complexity after protein enrichment and the multiple wash steps. After digestion the magnetic beads were removed and the digest was acidified, dried and reconstituted for LC-SRM readout of the target peptides. The chromatographic gradient was 30 min in these experiments, but a shorter gradient of 12 min was also successfully applied for improved sample throughput purposes (data not shown). Resulting data was analyzed in the Skyline software<sup>32</sup>. The chromatograms demonstrated very high reproducibility with 1.1-2.0 %CV in the chromatographic retention times, as well as in the general pattern of the signals of the different transitions and the correct peptide peaks could easily be identified as shown in Figure 3.

*Response curves* – Response curves were generated to monitor the linearity of the signal response over a concentration range of 5-1000 ng/ml (Figure 4-5). The two scFvs developed



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3 against each target protein were mixed before coupling to magnetic beads and used for  
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5 capture of the three target proteins (KRT19, MUC1-Fc and BRCA1) individually (Figure 4).  
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7 All peptides for all target proteins were easily detected down to the lowest concentration (5  
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9 ng/ml) with good linearity over the selected range. For the multiplexed captures, the magnetic  
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11 beads coupled with the scFv of different specificity were mixed and used to capture the three  
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13 target proteins over the same concentration range (Figure 5). Two out of the three peptides  
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15 used to monitor KRT19 were detected down to the lowest concentration and all three peptides  
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17 showed excellent linearity. The two peptides used to monitor MUC1-Fc were also detected  
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19 over the entire range with good linearity while the two BRCA1 peptides, although detectable  
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21 over the entire range demonstrated a somewhat poorer linearity. The curves were made in  
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23 triplicate from the very beginning of the protocol generating overall AFFIRM assay median  
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25 CVs between 7-18.2%, excluding one BRCA1 peptide with an outlying CV as summarized in  
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27 Table 1. The blank captures, i.e. captures with no spiked-in target proteins, gave a very low  
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29 degree of background signal indicating clean captures with a low degree of interferences. This  
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31 was also true for multicaptures as shown in Figure 3. Also, the peptides representing the scFv  
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33 and the three serum proteins that dominated the background signal gave very stable signals in  
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35 all captures (data not shown).  
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43 *Relative protein enrichment* - To estimate the relative enrichment achieved by the antibody  
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45 in the captures, the three target proteins were analyzed in a serum background with no prior  
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47 captures. A dilution series ranging from 5 ng/ml to 100 000 ng/ml was constructed to  
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49 determine the limit of detection (LOD) of the target proteins in serum. The samples were  
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51 digested, cleaned and analyzed using LC-SRM. KRT19 was barely detected at all in these  
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53 samples, only one peptide could be detected at a spiked-in concentration of 100 000 ng/ml. As  
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55 illustrated in Figure 4, all three of the target proteins could with AFFIRM easily be detected  
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down to 5 ng/ml, the lowest concentration tested. Thus, the limit of detection of the AFFIRM capture samples is 5 ng/ml at least, equaling an enrichment factor of at least 20 000 for KRT19. For BRCA1 both peptides were detected at a spiked-in concentration of 500 ng/ml, which equals an enrichment factor of at least 100. For MUC1-Fc the results were difficult to interpret for one of the peptides and the limit of detection in serum was 780ng/ml, whereas for the other peptide the detection limit was 12 500 ng/ml. This corresponds to an enrichment factor ranging between 156-2500.

## DISCUSSION

Sensitive and specific measurements of proteins of interest are greatly needed in many application areas. In the search and establishment of novel biomarkers for the clinic, lack of technology remains a major bottleneck and an intermediate verification step to move potential protein biomarkers from discovery studies towards true clinical qualification has been suggested<sup>34</sup>. This calls for technologies that meet several requirements such as sensitivity, specificity, multiplexing ability and throughput. In this work, we present the development of the novel immunoaffinity platform AFFIRM, combining targeted protein enrichment using scFvs with SRM readout for specific and sensitive protein analysis in both a single- and multiplexed format. The AFFIRM assay has been optimized to make the workflow fast and automated for increased throughput, reproducibility and cost efficiency.

AFFIRM takes advantage of the two powerful technologies recombinant antibody technology and MS. Both of these technologies are well established in the field of proteomics and the combination of the two has shown great capacity as a non-targeted fractionation strategy analyzing previously unexplored parts of the proteome<sup>35,36</sup>. This is, however, to the best of our knowledge, the first time that recombinant antibody technology has been combined with targeted MS readout through SRM analysis to create a platform for targeted protein enrichment and quantification in a multiplexed and flexible assay format.

SRM MS analyses have been demonstrated to reliably produce accurate measurements of target proteins of interest from complex biological samples such as tissue, cells or serum<sup>12,18,37-40</sup>. In addition, SRM can produce isoform specific readout<sup>8</sup> or information on post-translational modifications<sup>41</sup>. This is of central importance in any biological study trying to understand molecular interactions and signaling networks. In the search for novel biomarkers this discriminatory power provides unparalleled opportunities through the specific monitoring

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of proteins that are unique to the disease state and maybe even the drivers of the disease itself as in the case of cancer specific mutations<sup>8,9,23</sup>.

For any platform using immunoaffinity enrichment, antibody availability continues to be a bottleneck. However, with the advent of *in vitro* display technologies<sup>42</sup> it is now possible to rapidly generate monoclonal recombinant antibody fragments against virtually any antigen. In comparison to immunization-based methods, namely polyclonal antibodies and monoclonal antibodies (mAbs), which in the past have been the major source of specific binders for the use as capture reagents<sup>43-45</sup>, recombinant methods for binder generation have a number of advantages. For example, polyclonal antibody preparations contain a diverse population of target-specific and non-target specific antibodies and may suffer from batch-to-batch variations<sup>46, 47</sup>, thus requiring re-validation upon each production round. This is, in contrast to recombinant antibody fragments and mAb, which provide a continuous and renewable source of antibodies. Further, *in vitro* display techniques allow control over the selection process to a higher extent and the ability to enforce antibody fragments with desired properties by fine-tuning the selection approach. In addition, *in vitro* selection methods provide direct access to the antibody gene sequence, which is required for subsequent genetic modifications that might be needed to improve binder characteristics or assay compatibility.

Importantly, *in vitro* display systems enables for high-throughput generation of specific binders, towards a large number of biological markers at the same time, by the use of automated systems<sup>26-29</sup>. This capability has opened up for creating large collections of specific probes against virtually the entire proteome. Examples of such large-scale efforts are the EU funded initiative AFFINOMICS<sup>48</sup> and the NIH Commons Fund's Protein Capture Reagents Program in the USA<sup>49</sup>. The ultimate goal of these initiatives is to create large standardized collections of well-validated binding molecules to be available for the research

society. Once these target specific probes have been developed, they can easily be produced within a few days and used as a plug-and-play tool for immunoassays such as AFFIRM.

Targeted MS based immune-capture assays are designed to enrich either on protein- or peptide-level. In this work, the AFFIRM platform has been presented using protein enrichment, although, in principle, the workflow can also be applied on peptides. In our view, enrichment on protein level offers several advantages. The development of high quality binders for targeting peptides is generally more challenging than the development of protein antibodies. This can be explained by a lack of extensive three-dimensional structure of many peptides. From a target point of view, protein enrichment allows for detection of protein variants that could, as already mentioned, be the ultimate biomarkers. Without prior knowledge of these isoforms or modification this information can easily be over-looked using peptide enrichment. Protein enrichment also makes the assay more flexible, reliable and robust through multiple peptide readout for each target protein allowing the use of the best performing peptides in any assay setup. The BRCA1 protein enrichment in this work exemplifies this. Both BRCA1 peptides perform equally well in the single BRCA1 capture (Figure 4C), while one of the peptides clearly does not give satisfactory results in the multiplexed assay and should therefore be omitted for protein quantification in this setting (Figure 5C). Also capturing proteins rather than peptides has the advantage that only the captured fraction of the sample needs to be digested, thus allowing for scaling up the sample volume to achieve higher sensitivity<sup>23</sup>. Less amounts of expensive enzyme are required and the reduced complexity of the samples allows for much faster digestion (5 h compared to the 12-18h usually applied in complex mixtures). In addition, in this work the digestion after protein capture was performed with no reduction and alkylation, as this require no sample cleanup which saves both time and sample-processing steps that may introduce variability into the assay. This being said, peptide captures have been successfully implemented in many

studies<sup>20,21</sup> and is in some case the preferred choice, for example when targeting highly unstable proteins or proteins prone to denaturation<sup>22</sup>.

Mass spectrometry-based quantification often includes the use of heavy isotope labeled target peptides<sup>12,19,23,50,51</sup>. These are useful as internal standards both to aid in identifying the correct chromatographic peaks of the target peptides and for quantification using the ratio between the light analytical signal and the heavy signal of known concentration for each peptide. This is especially useful in the case of peptide centric capture experiments as the heavy isotope peptides can be introduced already before capture and therefore account for losses during enrichment. The digestion efficiency is however not controlled for. Heavy peptides can also aid in protein captures both for peak identification and as a means to normalize for when calculating run to run variation<sup>23</sup> and will be incorporated into the AFFIRM platform in the future for further quality control. The ideal internal reference for absolute quantification would be to spike samples with heavy isotope labeled target proteins to also account for losses in the digestion process<sup>51,52</sup>. Such labeled proteins are however very expensive.

The AFFIRM experiments provided detection of all three target proteins down to 5 ng/ml, which was the lowest concentration explored. The relative enrichment was however very spread with enrichment factors ranging between 100-20000 as the target proteins were differently well detected directly in spiked serum. This is probably due to a combination of factors, such as the digestion efficiency of the individual proteins in the complex sample mixture<sup>52</sup> and the affinity of the scFvs for their respective targets. The difference in detection of different peptides from the same protein, as seen with the two MUC-Fc peptides, also highlights how different peptides are differently accessible to MS analysis. For selected proteins, quantification down to hundreds of ng/ml has been reported by LC-SRM analysis alone<sup>53</sup>. Additional upfront depletion<sup>54</sup> and/or fractionation<sup>54,55</sup> can further improve

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3 detection limits to reach the high pg/ml to the low ng/ml range. Immunoaffinity enrichment  
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5 has been demonstrated to provide sensitivities in the low ng/ml range <sup>40,56</sup>. Importantly,  
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7 targeted immunoaffinity protocols provide a more selective enrichment and can make way for  
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9 MS analysis of inherently difficult proteins. In addition, immunoaffinity enrichment  
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11 efficiently reduces sample complexity to make it more easily adapted to fast and automated  
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13 analysis protocols <sup>57</sup>. To conclude, a concentration of 5 ng/ml of target protein detection is  
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15 well in the range demonstrated by other LC-MS based assays. However, experiments spiking  
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17 lower concentrations of the target proteins are needed to determine the true limit of detection  
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19 of the AFFIRM assay. We are confident that further improvements can be implemented in the  
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21 workflow to reach closer to the pg/ml sensitivity range reported by other immunoassays, e.g.  
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23 well-developed ELISA assays <sup>19</sup>. Such improvements include the use of well-characterized  
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25 antibody pairs, having higher affinity, better specificity and preferable binding to different  
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27 epitopes. Also, we believe that the washing procedure can be optimized in order to remove  
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29 potential non-specific interactions that may lead to loss in sensitivity. We are also working to  
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31 remove the drying step after the trypsin digestion in the current protocol. Digesting directly in  
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33 folie-covered 96-well plates and subsequent direct LC-MS injection would most likely both  
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35 lower variability and increase sensitivity of the assay.  
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41 In conclusion, we have developed a platform that allows for rapid development of novel  
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43 immunoaffinity LC-SRM assays through efficient selections of scFv antibodies for protein  
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45 capture and parallel development of LC-SRM assays for sequence based protein identification  
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47 and quantification. With this platform all requirements necessary for proteomics data to be  
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49 moved towards clinical assays are addressed; high sensitivity through specific antibody  
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**Figure 1.** The AFFIRM platform. Target-specific single chain antibody fragments and SRM assays are developed in parallel to create collections or libraries (A). This allows for fast implementation of the AFFIRM assay through production of scFv in 96-well format and coupling to magnetic beads (B). The assay is run in a 96-well format with a 1-h incubation of coupled beads in serum samples followed by washes, on-bead digestion and direct LC-SRM analysis without any further sample cleanup (C).

**Figure 2.** Specificity analysis of antibody fragments as determined by ELISA. Soluble recombinant antibody fragments C-Keratin19-1, C-Keratin19-3, MUC1-4, MUC1-5, BRCA1:10 and BRCA1:14 were investigated for their binding to recombinant protein (●) KRT19, MUC1-Fc and BRCA1, and negative control (■) BSA.

**Figure 3.** Extracted ion chromatograms of the BRCA1 target peptide EKPELTASTER captured exemplifying signal readout in single- (A-D) and multiplexed (E-H) AFFIRM assay format. The blank captures (A and E) show a very low degree of background signal in both single and multiplexed formats.

**Figure 4.** Response curves for the enrichment of target protein in a serum background using phage-selected scFvs. Curves are obtained by plotting the SRM signal peak area against the spiked-in target protein concentration. The response curves were generated for a protein concentration range of 5-1000 ng/ml. Enrichment of KRT19 using two scFvs (A), BRCA1 using two scFvs (B) and MUC1 using two scFvs (C) demonstrate that the target proteins can be detected down to the lowest concentration for all three proteins. The curves show very good linearity with a coefficient of determination ( $R^2$ ) of 0.99 for all three KRT19 peptides,



0.99 and 0.98 for the two MUC1 peptides while the two BRCA1 peptides provide slightly less linearity with R<sup>2</sup> values of 0.92 and 0.88 respectively.

**Figure 5.** Multiplexed response curves in triplicate with standard deviations for the enrichment of target protein in a serum background using phage selected scFvs plotting area under chromatographic curve in the SRM signal against target protein concentration. The response curves were generated for a protein concentration range of 5-1000 ng/ml using all six scFv to enrich for the three target proteins in the same capture experiment. Two out of three target peptides for KRT19 were detected down to the lowest concentration (5 ng/ml) (A) and both peptides for MUC1 (B) and BRCA1 (C) respectively were detected down to the lowest concentration. Average CVs are summarized in Table 1.

**Table 1.** The three target proteins measured, their target peptides measured and corresponding median CV in multiplexed AFFIRM analysis.

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ASSOCIATED CONTENT

**Supporting Information**

The mass spectrometry data and transition list has been deposited to the PeptideAtlas SRM Experiment Library PASSEL ([www.peptideatlas.org/passell](http://www.peptideatlas.org/passell)). Transition lists are also provided as supplementary table S1.

AUTHOR INFORMATION

**Corresponding Author**

Sofia Waldemarson, Department of Immunotechnology, Lund University, Medicon Village (House 406), SE-223 81 Lund, Sweden. Tel.: + 46 (0) 46 222 7469 (office). Email: Sofia.Waldemarson@immun.lth.se.

**Present Address**

<sup>1</sup> Department of Cell Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, GAC-1200; La Jolla, CA 92037, USA

<sup>2</sup>Department of Chemical and Systems Biology, Stanford School of Medicine, Stanford, CA, 94305, USA

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

<sup>‡</sup> These authors contributed equally

<sup>†</sup> These authors contributed equally

## Notes

The authors declare that there are no conflicts of interest.

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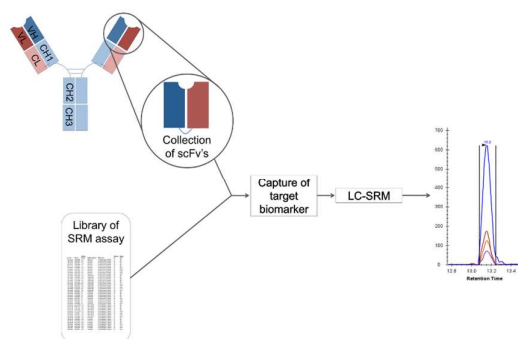
Table 1

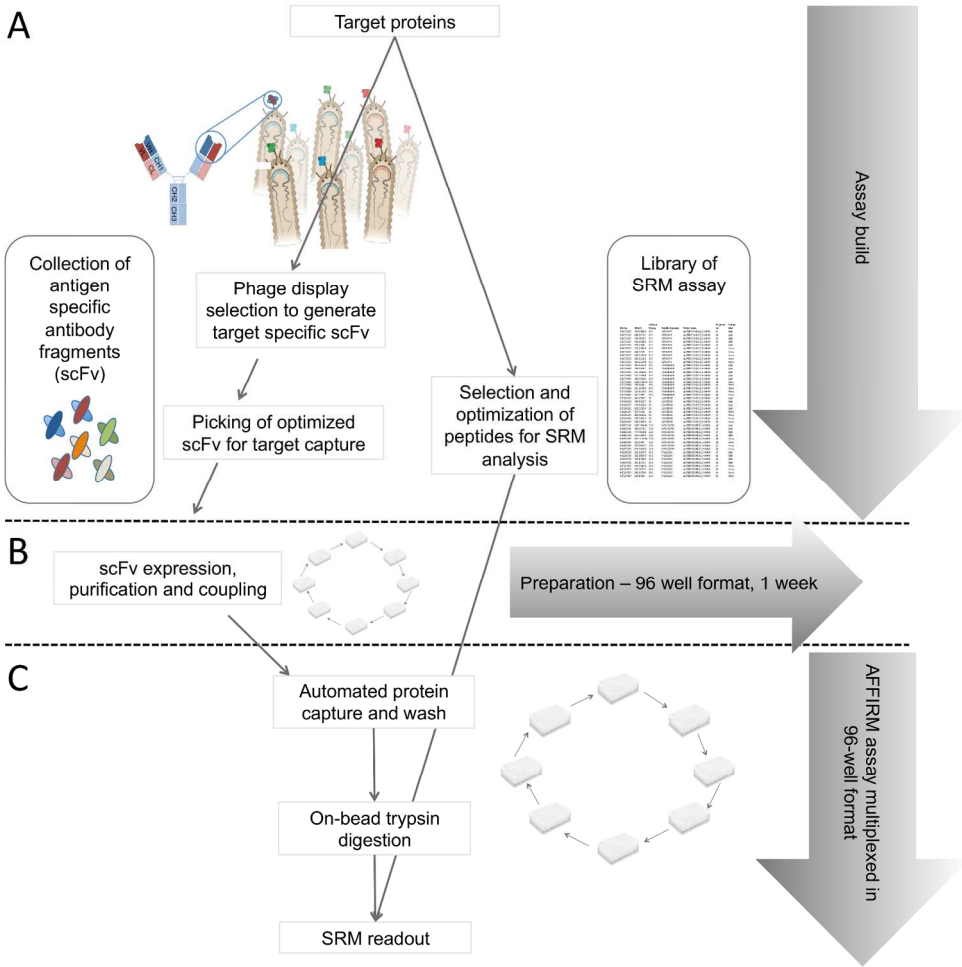
Protein Name	Protein ID / Uniprot Accession Number	Peptide Sequence Targeted	Median CV (%)
Keratin, type I cytoskeletal 19 (KRT19)	P08727	FGPGVAFR	13.6
		LTMQNLNDR	12.0
		ILGATIENSR	11.2
Breast cancer type 1 susceptibility protein (BRCA1)	P38398	EKPELTASTER	30.6
		YFLGIAGGK	18.2
Mucin-1-Fc* (MUC1-Fc)	P15941/P01863	DLPAPIER	7.0
		TELNYK	9.6

\* MUC1-Fc is analyzed in AFFIRM using scFv targeting MUC1 and an SRM readout of two peptides from the Mouse IG gamma-2A chain C region

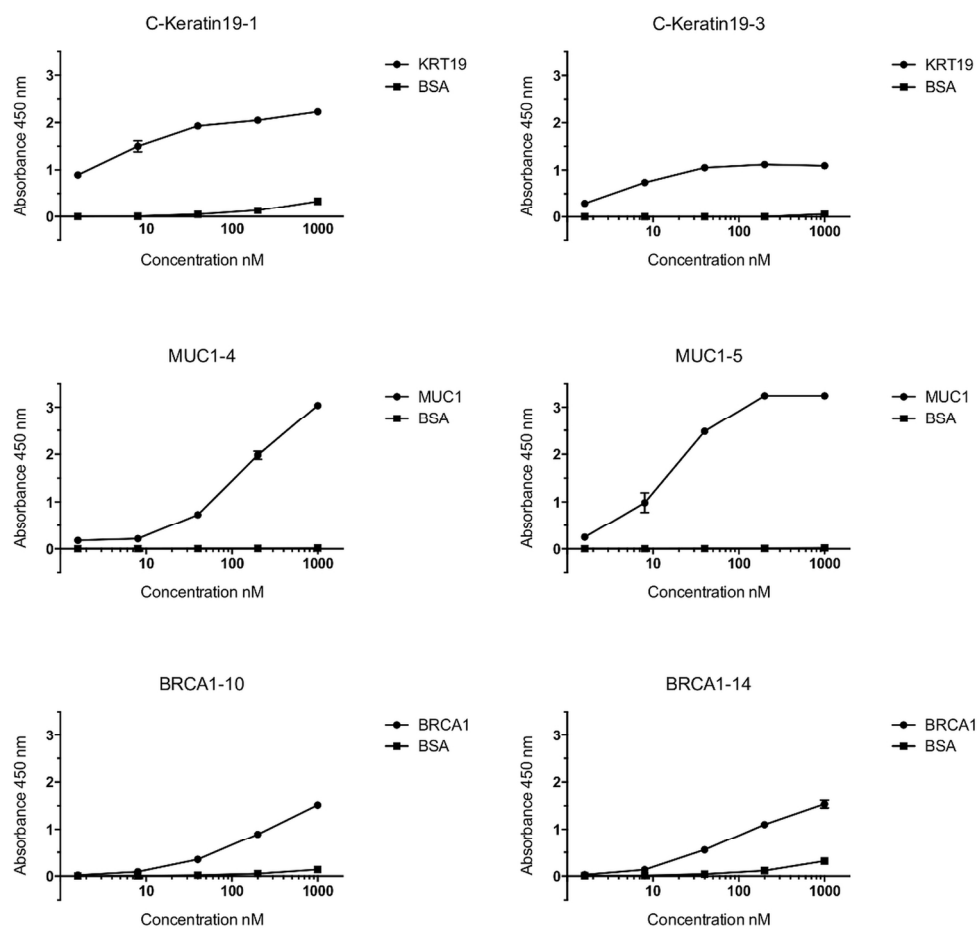


## Abstract Graphic

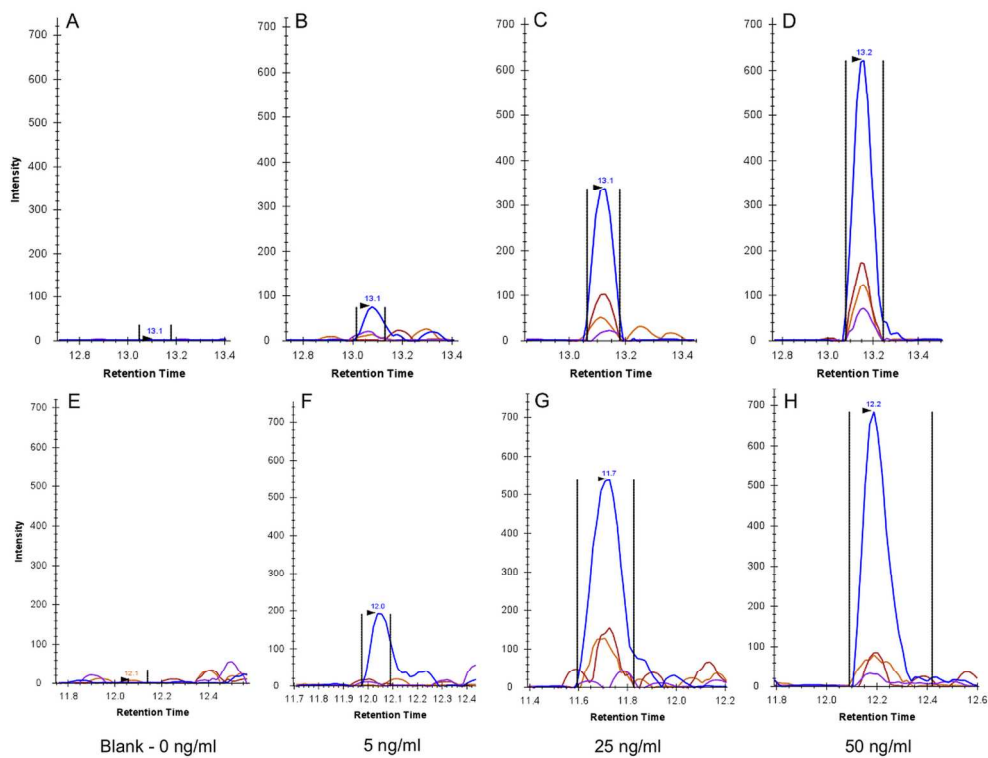




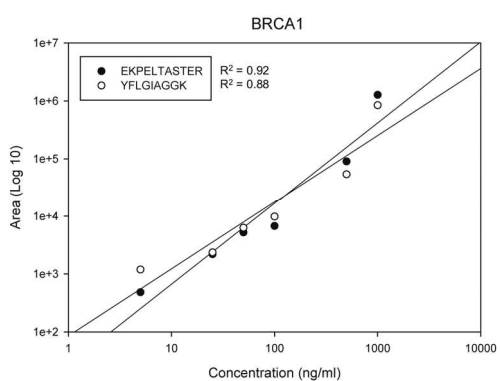
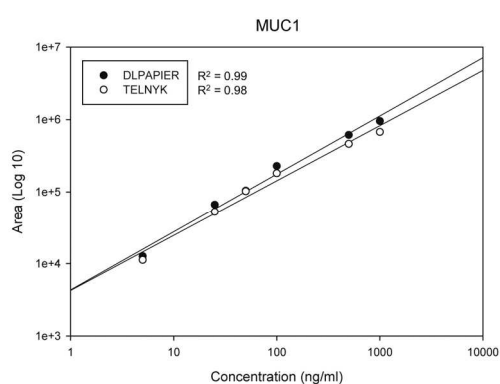
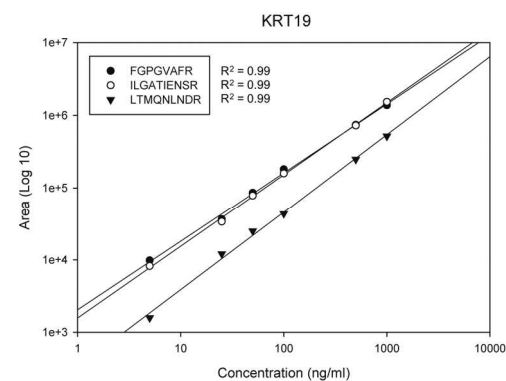
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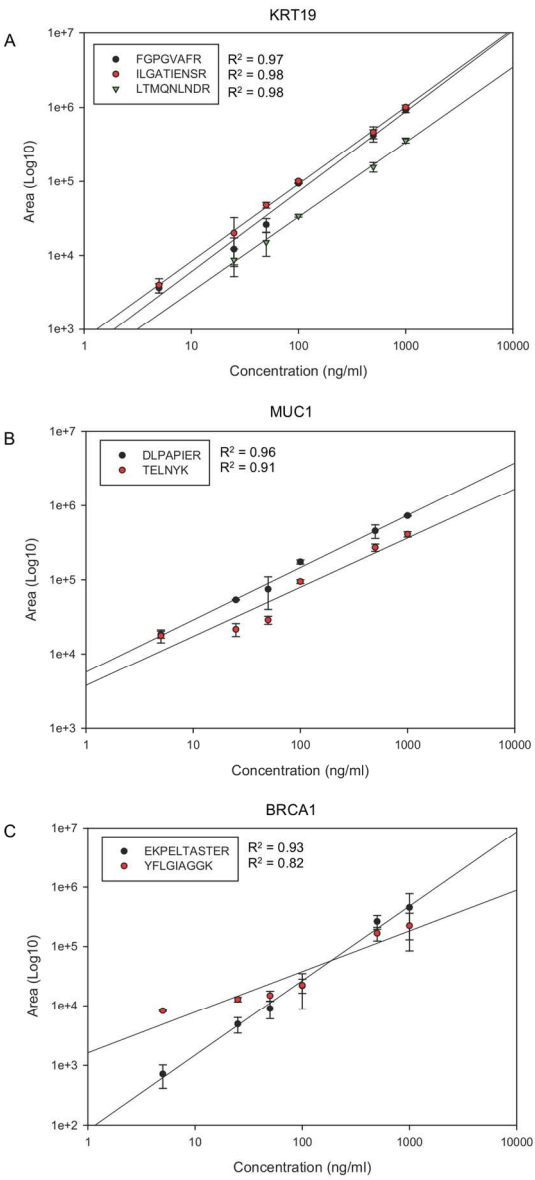
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159x122mm (300 x 300 DPI)



84x193mm (300 x 300 DPI)



84x180mm (300 x 300 DPI)