Research Use Only Immunoassays for Early Detection of Breast Cancer

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❶ Abstract

Through previous cycles of Early Detection Research Network (EDRN) funding, the Fred Hutchinson Breast Cancer EDRN Clinical Validation Center led by Drs. Li and Partridge identified the following candidate biomarkers for early detection of estrogen receptor positive breast cancer: Reg-4, Jak-2, and IgG and/or IgM autoantibodies to TLR-2, Cux-1 and mesothelin. Markers were discovered using an array containing more than 3,000 antibodies to more than 2,000 proteins. Protein biomarkers were discovered by labeling samples from multiple high-quality cohorts with Cy5 (635nm) and comparing to a reference pool labeled with Cy3 (532nm). Antigen/autoantibody complexes were discovered using Alexa Fluor labeled goat anti-human IgG or IgM antibody. To further validate these findings, assays for selected biomarkers need to be developed using a complementary, robust and high throughput technology. Here we present initial results of developing Research Use Only (RUO) Meso Scale Diagnostics, LLC. (MSD) MULTI-ARRAY immunoassays for these targets. All serum and plasma samples used for this development work were commercially sourced.

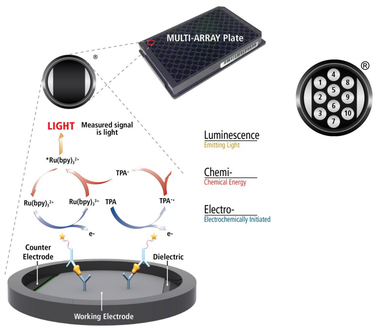
Multiple antibodies targeting Reg-4 were screened in a sandwich immunoassay format with recombinant and native Reg-4 and serum and plasma samples. An antibody pair was selected based on sensitivity, spike recovery and dilution linearity data. The assay was sufficiently sensitive to measure Reg-4 in 100-fold diluted serum and plasma, and Reg-4 levels in matched plasma and serum samples correlated well. Median concentration of Reg-4 was higher in 40 samples from individuals with breast cancer than in samples from approximately 100 apparently healthy controls.

Preliminary data from developing a sandwich immunoassay for JAK-2 are also presented.

Recombinant Cux-1, TLR-2, and mesothelin were immobilized on MULTI-ARRAY plates and incubated with diluted serum or plasma 2,500-fold dilution for IgG measurements). IgG autoantibodies were detected using MSD SULFO-TAG™ labeled anti-human antibodies. Initial results with samples from apparently healthy individuals and individuals with breast cancer are presented.

❷ Methods

MSD® electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.



MULTI-ARRAY Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

❸ Serology Assays

Autoantibodies against Cut like homeobox 1 (Cux-1), mesothelin, and Toll-like receptor 2 (TLR2) have been identified by the Fred Hutchinson Breast Cancer EDRN Clinical Validation Center as potential biomarkers for early detection of breast cancer.

A multiplexed research-use-only serology panel was developed to detect autoantibodies against Cux-1, TLR-2, and mesothelin. Plates are provided with antigens arrayed within the wells of a 96-well plate. Antibodies in the sample bind to the arrayed antigens, and anti-human IgG antibody conjugated with MSD SULFO-TAG is used for detection. Commercially sourced plasma or serum samples were tested at 2,500-fold dilution on the serology panel.

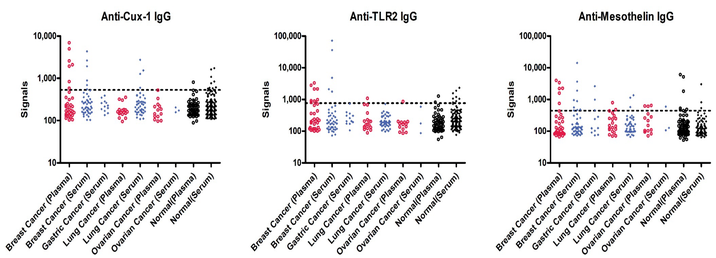


Figure 1. ECL signals for assays detecting autoantibodies against Cux-1, TLR2 and mesothelin, in commercially sourced samples. Dashed black lines show the 95th percentile of signals from 197 serum and plasma samples from apparently healthy individuals. Using this 95th percentile cutoff, 16% of samples from individuals with breast cancer had elevated autoantibodies against Cux-1, 18% against TLR2, and 19% against mesothelin.

❹ Reg-4 Sandwich Immunoassay Development

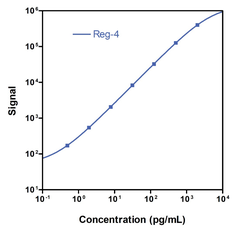
Regenerating islet-derived type 4 (Reg-4), a member of the calcium-dependent lectin gene superfamily, is abnormally expressed in various cancers, such as colorectal, gastric, gallbladder, pancreatic, ovarian, prostate, and lung cancer. Serum or plasma concentrations of Reg-4 protein may be elevated in early stage breast cancer.

Eight antibodies against Reg-4 were screened in all combinations against recombinant Reg-4 expressed in either *E. coli* or a mammalian system, and against pooled breast cancer serum. Twelve promising antibody pairs were further evaluated. The table below shows results of a calibration curve and a diluted, pooled breast cancer serum sample. Multiple antibody pairs produced good calibration curves with Hill slopes of less than 1.1 and detection limits of around 1 pg/mL. The four-fold diluted, pooled breast cancer serum sample had a Reg-4 concentration of approximately 1.4 ng/mL indicating that the assay is sufficiently sensitive to allow one to dilute serum or plasma significantly, as needed. The twelve antibody combinations measured Reg-4 at a similar level in the pooled breast cancer sample, giving confidence that each of the candidate assay formats accurately measures Reg-4.

Subsequent experiments included optimizing critical reagent concentrations, selecting assay diluents, and assessing dilution linearity and spike recovery. The best antibody pair was selected based on these results.

Capture Ab	AB5	AB4	AB6	AB7	AB5	AB4	AB7	AB4	AB6	AB5	AB7	AB6
Det.Ab	AB4	AB5	AB2	AB4	AB2	AB2	AB2	AB3	AB3	AB6	AB3	AB5
[Ag] pg/mL	ECL Signal											
10,000	1,876,959	1,489,483	1,793,897	1,724,794	1,691,266	1,959,799	1,865,347	1,670,137	1,432,265	822,886	1,534,427	1,226,231
2,500	802,915	468,076	508,480	614,357	474,500	785,794	577,083	458,823	346,969	237,028	381,306	366,682
625	229,859	134,254	122,363	173,022	123,747	168,236	123,241	248,863	61,704	65,803	60,732	107,356
156.3	57,230	33,333	28,431	42,557	24,597	34,094	24,040	13,949	9,827	16,663	8,733	27,446
39.1	13,794	8,264	7,279	10,129	6,476	8,023	5,731	3,608	2,701	4,615	1,763	7,803
9.8	3,427	2,084	1,902	2,663	1,541	2,019	1,590	949	673	1,520	424	2,975
2.4	1,003	544	609	728	597	573	493	263	234	672	144	1,686
0	78	58	145	99	101	72	112	59	88	420	48	1,266
Hill Slope	1.02	1.01	1.02	1.02	1.02	1.08	1.07	1.13	1.14	0.99	1.23	0.99
Estimated LOD (pg/mL)	0.3	0.4	0.6	0.4	0.6	0.5	0.8	1.3	1.9	2.0	2.8	3.7
4-fold diluted Pooled Breast Cancer Serum (pg/mL)	1,368	1,296	1,384	1,712	1,244	1,371	1,338	1,390	1,469	1,509	1,242	1,341

Table 1. ECL counts for a calibration curve and calculated concentration of a pooled sample for candidate antibody pairs.



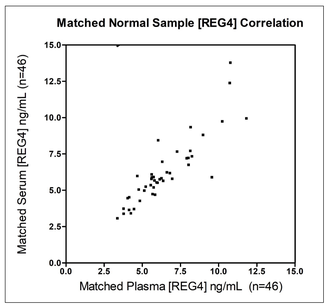
Sample Dilution Factor	100
Top Calibrator (TOC)	2,000 pg/mL
Limit of Detection (LOD)	0.3 pg/mL
Dilution Adjusted TOC	200 ng/mL
Dilution Adjusted LOD	30 pg/mL
Hill Slope	1.02

Protocol

1. Add calibrator, control, or 100-fold diluted sample to assay plate (25 µL/per well). Incubate 1 hour at room temperature (RT).
2. Wash and add detection antibody solution (25 µL per well). Incubate 1 hour at RT.
3. Wash plate and add MSD GOLD™ Read Buffer B (150 µL per well). Analyze with MSD instrument.

Figure 2. A typical Reg-4 calibration curve. The assay has a wide dynamic range of four orders of magnitude. Only 25 µL of a 100-fold diluted serum or plasma sample is required per replicate. The assay protocol is simple and can be completed in three hours.

❺ Individual Serum and Plasma Sample Testing



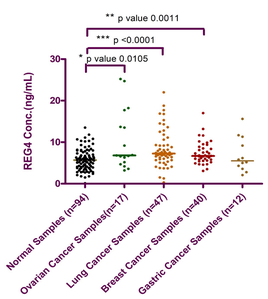


Figure 3. The research-use-only Reg-4 assay was evaluated using the following commercially sourced samples: 140 serum or plasma samples from apparently healthy individuals and 126 serum or plasma samples from individuals with lung, breast, gastric or ovarian cancer. Serum and plasma samples from the same donor gave similar Reg-4 concentrations (graph on left). Reg-4 concentrations were elevated in several cancer samples compared to the apparently healthy controls. P-values were calculated using the Mann-Whitney test (graph on right).

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❻ Initial JAK2 Immunoassay Development

Serum/plasma Janus kinase 2 (JAK2) has been identified by the Fred Hutchinson Breast Cancer EDRN Clinical Validation Center as a potential biomarker for early detection of breast cancer. To develop a sandwich immunoassay against JAK2, more than 60 antibodies were screened in all combinations with recombinant JAK2, a pooled serum sample, cell lysate and a zero calibrator condition using MSD's high-throughput MULTI-ARRAY technology. The two figures below show heat maps of background-subtracted signal for calibrator (top) and serum pool (bottom). Capture antibodies are shown in rows, and detection antibodies in columns. Observed signals are shown graded with highest signals highlighted in green and low signals highlighted in red. Antibodies showing similar signal patterns – presumably because they bind to similar epitopes – were grouped together and each presumed epitope group is color-coded.

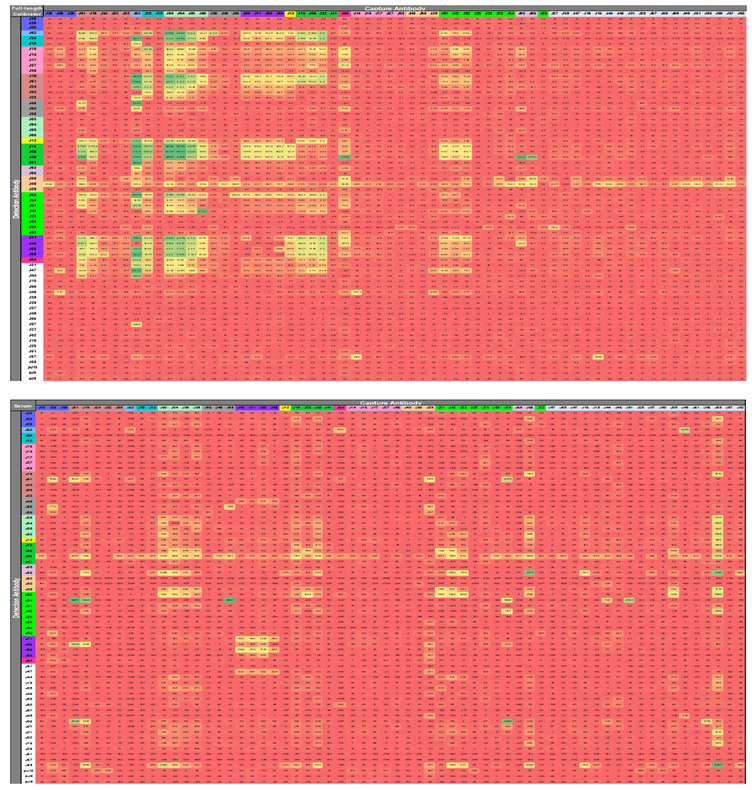

Figure 4. Antibody screening heat map for recombinant JAK2 (top) and a serum pool (bottom). Several antibody pairs gave high signals for both recombinant calibrator and the serum pool. These promising antibody pairs will be further investigated.

❼ Conclusions

- The Fred Hutchinson Breast Cancer EDRN Clinical Validation Center identified candidate serum/plasma biomarkers for early detection of breast cancer. Here we show development of immunoassays against selected targets using a complementary, robust and high throughput technology.
- We completed initial assay development for autoantibodies to Cux-1,TLR2, and mesothelin.
- We successfully developed a research use only sandwich immunoassay for Reg-4. The assay has a wide dynamic range and high sensitivity, requiring only 25 µL of a 100-fold diluted serum or plasma sample.
- We began development of a sandwich immunoassay for JAK2. Initial data are promising.

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❽ Acknowledgements

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