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Development of a three-plex single molecule immunoassay enabling measurement of the EGFR ligands amphiregulin, betacellulin and transforming growth factor α simultaneously in human serum samples.

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Abstract

Background
Prior to large studies in breast cancer patients and healthy individuals we established a sensitive three-plex immunoassay to measure the EGFR ligands amphiregulin (AR), betacellulin (BTC) and transforming growth factor α (TGF-α) simultaneously in human serum samples.

Method
The three-plex immunoassay was developed using single molecule array (Simoa) technology and requires only 20 µl of serum.

Results
AR, BTC and TGF-α were first established as three single-plex assays. Multiplexing the three single-plex assays showed no significant cross reactivity between the reagents. The concentrations of the ligands in serum samples showed correlations $r^2 \geq 0.84$ between the single-plex and three-plex methods. The three-plex assay demonstrated limit of detection levels at 0.16 ng/L for AR, 0.23 ng/L for BTC and 0.22 ng/L for TGF-α. Total coefficients of variations were 8.5%-31% for AR, 11%-21.8% for BTC and 12.4%-16.2% for TGF-α. Spiking experiments showed a mean recovery of 97% for AR, 86% for BTC and 81% for TGF-α. The concentrations of the EGFR ligands did not change significantly after series of freeze thaw cycles or incubation at 22°C for up to 24h.

Conclusion
This robust three-plex assay with up to 40-fold increase in sensitivity relative to conventional ELISA is the first published method that has the required sensitivity to measure AR, BTC and TGF-α simultaneously in human blood samples.

Keywords: Single molecule array; Simoa; digital ELISA; multiplexed immunoassays; EGFR ligands
Abbreviations

Simoa, Single molecule array; TGF-α, Transforming Growth factor α; AR, Amphiregulin; BTC, Betacellulin; EGFr, Epidermal Growth factor Receptor; SβG, Streptavidin-β-galactosidase; RGP, resorufin-β-D-galactopyranoside; AEB, average number of enzyme per bead; LOD, lower limit of detection; CV%, coefficient of variation.
1. Introduction

AR, BTC and TGF-α are three of seven ligands for EGFr. Upon ligand binding EGFr form homo/heterodimeric complexes with HER2 as the preferred dimerization partner (1). This enables transduction of a biological signal from the outside to the inside of the cell, which leads to activation of intracellular signaling cascades involved in various cell responses including cell proliferation, angiogenesis and resistance to apoptosis. Misregulation of either EGFr itself or its ligands is implicated in the pathogenesis of several types of cancer including lung, ovarian, colorectal and breast cancer. EGFr and some EGFr ligands are known to be expressed differently in tumor tissue as compared to healthy tissue in several types of cancer and levels are known to be associated to prognosis (2-4). In lung cancer and colorectal cancer there are indications that levels of AR and TGF-α in the blood can provide valuable information regarding prognosis and prediction of response to certain EGFr-targeted treatments (5-7). In breast cancer the predictive and prognostic value of EGFr and its ligands in blood remain to be investigated in detail (8).

As we intend to investigate the prognostic and predictive information provided by concentrations of EGFr ligands in blood in breast cancer we attempted to measure AR, BTC and TGF-α in serum samples using commercial available immunoassays. However, we were not able to achieve measurable results using serum samples from either healthy individuals or from breast cancer patients. To our best knowledge there are no publications that describe measurement of BTC concentrations in the blood. AR and TGF-α have been investigated in serum from both cancer patients and healthy individuals. Common for these studies is insufficient assay sensitivity for quantifying the ligands especially in samples of healthy individuals but also in cancer patients thus 50-100% of samples have concentrations below the first calibrator (9;10) (5;11). Furthermore commercial available immunoassays with low calibrator levels at 15.6 ng/L for measuring TGF-α and AR describe in the kit inserts that the serum level in healthy individuals is detectable in only 30% and 3% of the samples (RnD Systems, Minneapolis, MN, USA). Thus higher assay sensitivity is definitely needed for measuring these ligands in serum than the traditional immunoassays can provide. The Simoa technology has shown up to a 1000-fold improvement in sensitivity as compared to conventional ELISA approaches (12;13). Besides the increased sensitivity this technology also has the advantage that assays can be multiplexed which is very valuable in projects with low volume of sample material.

The purpose of this study was to develop an assay with sufficient sensitivity using the Simoa technology to quantitate AR, BTC and TGF-α simultaneously in serum samples.
2. Materials and methods

2.1. Simoa
The development of the three-plex assay was performed on the automated Simoa HD-1 Analyzer platform (Quanterix®, Lexington, MA, USA). This instrument uses the same reagents as conventional ELISA but uses femtoliter-sized reaction chambers approximately 2 billion times smaller than conventional ELISA. This will result in a rapid buildup of fluorescence if a labeled protein is present and making it possible to detect single molecules. The instrument has been described in detail (14).

2.2. Reagents
Antibodies (AR cat. No. MAB262; BTC cat. No. AF261 and TGF-α cat. No. AF239, R&D Systems, Minneapolis, MN, USA) were covalently attached by standard carbodiimide coupling chemistry to carboxylated paramagnetic beads (Homebrew Multiplex Beads, Quanterix (488 L1-AR), (750 L1.5-BTC), (700 L1.5- TGF-α). The biotinylated detector antibodies were from R&D Systems (AR cat. No. BAF262, BTC cat. No. BAF261 and TGF-α cat. No. BAF239). The calibrators were recombinant proteins from R&D Systems (AR cat. no. 262-AR-100; BTC cat. no. 261-CE-010 and TGF-α cat. no. DY239). Streptavidin-β-galactosidase (SβG), enzyme substrate resorufin-β-D-galactopyranoside (RGP) and all consumables including wash buffers, cuvettes, disposable tips, and discs were from Quanterix.

2.3. Simoa protocol
The three-plex assay is a 2-step assay. Before running the following reagents are prepared; the single-plex beads are mixed equal in bead diluent buffer (Quanterix) and diluted to a final concentration of 2.0*10⁷ beads/mL. The biotinylated detector antibodies are mixed and diluted in sample/detector diluent (Quanterix) to final concentrations of 0.2 mg/L for AR, 0.04 mg/L for BTC and 0.06 mg/L for TGF-α. The SβG is diluted in SβG diluent (Quanterix) to 100 pM. After loading the prepared reagents and consumables, the calibrators are prepared in diluent A (Quanterix) with 10μg/mL Superchemiblock Heterophile Blocking Agent (EMD Millipore, Darmstadt, Germany). The AR, BTC and TGF-α calibrators are mixed to final concentrations of 300 ng/L for each followed by a 3-fold titration and together with neat samples and controls they are loaded onto the instrument in a 96-well microtiter plate. In all experiments the calibrators, samples and controls are
run in duplicates. The following steps are performed by the instrument. First 25 µL of multiplex bead mixture is pipetted into a cuvette together with 80 µL of sample, control or calibrator and 50 µL mixture of biotinylated detection antibodies. The instrument performs a 4-fold dilution of the samples and controls in diluent A with 10µg/mL Superchemiblock Heterophile Blocking Agent. An incubation step for 30 min is performed and the beads are then magnetically separated and washed. 100 µL of SβG is then added to the cuvette by the instrument and incubated for 5 minutes. The beads are then separated magnetically and washed following the addition of RGP substrate. The bead substrate mixture is then loaded on to the Simoa disc containing an array of 216,000 microwells and sealed with oil. If AR, BTC or TGF-α has been captured and labeled, the SβG hydrolyze the RGP substrate into a fluorescent product that can be measured. At low concentrations of proteins, beads carry either zero or low numbers of enzymes and protein concentration is quantified by counting the presence of “on” or “off” bead (digital). At higher concentration of protein, each bead carries multiple enzymes and the total fluorescence signal is proportional to the amount of protein in the sample (analog). Both the digital and analog calculations use the unit: average number of enzyme per bead (AEB). The concentrations of AR, BTC and TGF-α in the unknown samples are interpolated from the calibrator curves obtained by 4 parameter logistic regression fitting.

2.4. Samples and controls
Serum samples from healthy individuals (n=12) and breast cancer patients (n=20) were applied for the comparison of the single-plex and three-plex methods. Blood was withdrawn and left 30 minutes for coagulation. Afterwards the samples were centrifuged for 10 minutes at 2000 G. The supernatant was transferred into tubes and stored at -80°C until use.
To investigate the stability of AR, BTC and TGF-α in blood samples, serum from 5 donors were frozen and thawed (f/t) for up to 5 cycles or left at 22°C for 2 h or 24 h before freezing.
Controls at various levels were prepared using serum for a low control and spiked serum for an intermediate and a high control. The controls were used to determine intra-assay and total coefficients of variations (CV%) and were included in each run.
3. Results

3.1. Setting up AR, BTC and TGF-α on the Simoa as single-plex assays.

Figure 1 shows the calibrator curves of the AR, BTC and TGF-α single-plex assays developed on the Simoa. In order to achieve low backgrounds and high signal to noise ratios various parameters were optimized for each assay. Concentrations ranging from 0.01 to 0.6 mg/L of detection reagent and from 50 to 150 pM of SβG, were tested. Furthermore several sample and calibrator diluents were examined and different running programs (2-step vs 3-step and minutes of incubations) for the Simoa instrument were also tested. The optimized concentrations of detector and SβG reagents, sample and calibrator diluents and assay parameters used for the single-plex assays are identical to the three-plex assay which is described in the material and method section 2.3. The final parameters are described in the materials and methods.

3.2. Setting up AR, BTC and TGF-α on the Simoa as a three-plex assay.

For multiplexing the three single-plex assays, experiments were performed to ensure that calibrators, detection reagents and capture beads do not cross-react and result in false positive signals. An AR calibration curve was performed based on its specific capture and detection reagents. It was compared with 1) the three-plex mixture of detection antibodies 2) the three-plex mixture of calibrators (Figure 2A). As shown in the figure no significant cross reactivity by any of the other ligands or detection reagents was observed in the AR assay. Similar experiments were carried out for the BTC and TGF-α assays (Figure 2B and 2C). For both assays there was a 2-3 fold increase in background signals using the three-plex mixture of detection reagents.

In order to test the specificity of the beads the three-plex capture beads and the three-plex detection reagents were applied. Using the AR calibrator alone resulted in a bead response from the AR beads (figure 3A). There was no bead response from either the BTC or the TGF-α beads below 100 ng/L, however, at higher concentration a slight increase in bead response from both BTC and TGF-α beads were observed. Using BTC calibrator alone resulted in a bead response from the BTC beads (figure 3B) and no bead response from the TGF-α beads but the AR beads responded slightly at concentrations above 100 ng/L of BTC calibrator. Using the TGF-α calibrator alone resulted in a bead response from the TGF-α beads and a slight bead response from the AR beads above 100 ng/L of TGF-α calibrator (figure 3C). The calibrator curves of the three-plex assay are shown in figure 4.
3.3. Validating the three-plex assay (dilution linearity, LOD, LOQ and CV%).

Dilution linearity: In order to measure matrix effect, two serum samples were spiked with 150 ng/L of AR, BTC and TGF-α recombinant proteins and then serial diluted in sample diluent buffer ranging between 2 and 16 fold (2, 3, 4, 6, 8, 16). The recovery mean ranges were (89-97%) for AR, (76-87%) for BTC and (64-91%) for TGF-α. The use of a 4 fold dilution which was used for further experiments resulted in a mean recovery of 97% for AR, 86% for BTC and 81% for TGF-α.

Lower limit of detection (LOD): LOD was determined using 3 SD from the blank (sample diluent buffer). The LOD was calculated for each of 5 runs across different days from duplicate measurements of the blank and estimated as the mean of the five experiments. The mean LOD for AR was 0.16 ng/L and 0.2 ng/L for TGF-α and BTC.

Controls, intra-assay CV% and total CV%: For determining the intra-assay CV% the three controls were run in replicates of 12 in one assay which resulted in the following taking the four fold dilution into account: Low control: AR mean 3.4 ng/L CV% 17.0%, BTC mean 4.0 ng/L CV% 7.9%, TGF-α mean 5.9 ng/L CV% 11.2%. Middle control: AR mean 13.6 ng/L CV% 8.3%, BTC mean 9.4 ng/L CV% 5.5%, TGF-α mean 12.4 ng/L CV% 5.9%. High control: AR mean 131 ng/L CV% 5.8%, BTC mean 99.7 ng/L CV% 7.9%, TGF-α mean 88.8 ng/L CV% 5.3%.

The total CV% was calculated from 6 runs across multiple days resulting in the following taking the four fold dilution into account: total CV% between 8.5% – 31% for AR, 11% – 21.8% for BTC and 12.4% – 16.2% for TGF-α. Low control: AR mean 3.5 ng/L CV% 31%, BTC mean 4.6 ng/L CV% 22%, TGF-α mean 6.2 ng/L CV% 16%. Middle control: AR mean 14 ng/L CV% 17%, BTC mean 10 ng/L CV% 20%, TGF-α mean 13 ng/L CV% 12%. High control: AR mean 130 ng/L CV% 9%, BTC mean 102 ng/L CV% 11%, TGF-α mean 92 ng/L CV% 13%.

LOQ: Allowing for a total CV% of 20% according to the Clinical and Laboratory Standard Institute, the LOQ was estimated by interpolation to be 2.5 ng/L for AR and BTC and 1.25 ng/L for TGF-α.

3.4. The single-plex assay versus the three-plex assay.

The concentrations of AR, BTC and TGF-α in serum samples from healthy individuals and breast cancer patients were determined using both the single-plex and three-plex methods (figure 5 ABC). For AR, the correlation was $r^2$=1.0 between the two methods and the slope of linear regression=0.82. For BTC the correlation was $r^2$= 0.84 and the slope of linear regression= 0.41 after excluding three high outliers. For TGF-α, the correlation was $r^2$=0.94 and the slope of linear
regression was = 0.95. The serum concentrations using the three-plex and the single-plex methods are depicted in table 1 as medians and concentration ranges. All serum samples had AR (n=32) and TGF-α (n=27) concentrations exceeding the first calibrator level at 0.4 ng/L using the three-plex assay while 2 of 32 samples had BTC concentrations below.

3.5. The stability of AR, BTC and TGF-α in serum samples.

To investigate the stability of AR, BTC and TGF-α in blood samples, serum was applied from 5 donors and subjected to series of freeze thaw cycles or storage at 22°C (Figure 6ABC). A Wilcoxon Signed-Rank Test for differences in medians showed no significant differences and all three ligands are thus highly stable in serum for up 5 f/t cycles and after incubation at 22°C for up to 24 h before freezing.
4. Discussion
The purpose of this study was to develop and validate a multi-plex assay with sufficient sensitivity to measure AR, BTC and TGF-α simultaneously in human serum samples. In order to develop the multi-plex assay it was first necessary to establish three single-plex assays. This was done by finding well performing antibodies and optimal condition for all parameters in the assays. The reason for developing a multi-plex method instead of applying the three single-plex assays was primarily in order to save time, consumables and valuable sample material. With this three-plex assay, results of AR, BTC and TGF-α can be achieved using only 20 µL of serum. Measuring the ligands using single-plex the serum requirement would have been 60 µl in total. Though this extra volume used for single determinations is not much it is a disadvantage in a research setting where access to sample material is often limited. Moreover the importance of performing multi-plex would in a clinical situation enable a much shorter turn-around-time of test results to clinicians which is getting more and more important in daily practice and in addition to save time and consumables. With this three-plex assay results of AR, BTC and TGF-α can be achieved using only 20 µL of serum.

The cross reactivity and specificity was investigated. No significant cross reactivity was observed in the AR assay, however, in the BTC and TGF-α assays a ~2-3 fold increase in background signals were observed when the three-plex mixture of detection antibodies were applied. This was highly expected from the use of a higher total concentration of detection reagents. The background signals of the three-plex assay are below 0.01 AEB for all ligands which is very acceptable.

In order to test the specificity of the capture antibodies the bead responses were tested. Especially the AR beads responded at concentration higher than 100 ng/L of either BTC or TGF-α calibrator. Also responses from the BTC and TGF-α beads were observed using AR calibrator concentrations above 100 ng/L. We expect the concentrations in the majority of serum samples to be below 100 ng/L i.e. 400 ng/L according to the 4-fold dilution used in this study however, two serum samples had a BTC concentration above that limit. All though false bead responses were minimal serum samples with concentrations exceeding 100 ng/L could be diluted further to prevent possibly false bead responses.

Regarding AR and TGF-α, there was a fine correlation between the serum concentrations determined using the single-plex and the three-plex methods. The correlation and slope of linear regression for BTC between the two methods was not as good as AR and TGF-α. The absolute measured concentration of BTC is biased by minus 50 % using multi-plex and hence can be
corrected for using a correction factor. However 95% of the samples included in this study were measurable using multi-plex so the assay is able to detect increased levels and differences in the normal range and the BTC performance as a three-plex assay is acceptable for our use. If used in a clinical situation in single patients the assay should be standardized and optimized further. The reason for that is unclear and remains to be investigated.

The stability of the AR, BTC and TGF-α in serum samples was investigated. All the ligands were found to be highly stable which is in accordance with the literature (10).

To our knowledge we are the first to develop a quantitative assay with the sufficient sensitivity to measure AR, BTC and TGF-α in human serum samples. The three-plex assay was able to measure AR, BTC and TGF-α in all of the serum samples obtained from both healthy individuals and breast cancer patients and BTC in 94% of the samples. By using the Simoa platform instead of a traditional ELISA we manage to increase the sensitivity 40-fold.

In conclusion we developed a stable and sensitive three-plex protein assay to measure AR, BTC and TGF-α simultaneously in serum samples. In the future we are planning to use this three-plex assay to establish AR, BTC and TGF-α reference intervals in healthy individuals and measure the ligands in serum from human breast cancer patients.

Declaration of interest
None

Funding
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Reference List

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Figure legends

Figure 1. AR, BTC and TGF-α calibrator curves run as single-plex assays. The average number of enzymes per bead (AEB) against concentrations is shown. The calibrators (n=5-6) were run in duplicates and the mean value for each point of the calibrators is shown.

Figure 2ABC. Reagent cross reactivity was determined using single-plex capture beads, single-plex detector reagents and single-plex analyte and compared to single-plex capture beads, three-plex detector reagents and three-plex analyte.

Figure 3ABC. The capture bead responses were tested using mixed capture beads, mixed detection reagents and one analyte at a time. The specific capture beads responded and below 100 ng/L of analyte no bead responses were observed from the other capture beads.

Figure 4. AR, BTC and TGF-α calibrator curves run as a three-plex assay. The average number of enzymes per bead (AEB) against concentrations is shown. The calibrators (n=8) were run in duplicates and the mean value for each point of the calibrators is shown.

Figure 5ABC. Plots showing correlation between concentration of AR, BTC and TGF-α in serum samples using the single-plex assay and the three-plex assay.

Figure 6ABC. The stability of AR, BTC and TGF-α in serum from five donors was tested under various conditions including one to five freeze and thaw cycles (1x f/t, 2x f/t, 3x f/t and 5x f/t) and incubation for 2h and 24h at 22°C. The medians are shown in the figure.
Table 1. The serum concentrations of the ligands measured using the single-plex and the three-plex assays.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Sample number</th>
<th>Three-plex Median (range) ng/L</th>
<th>Single-plex Median (range) ng/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>32</td>
<td>7.2 (2.96-187)</td>
<td>9.3 (2.43-220)</td>
</tr>
<tr>
<td>BTC</td>
<td>32</td>
<td>2.74 (0.1-1504)</td>
<td>6.2 (1.0-2456)</td>
</tr>
<tr>
<td>TGF-α</td>
<td>27</td>
<td>3.65 (1.3-6.57)</td>
<td>3.2 (1.3-7.0)</td>
</tr>
</tbody>
</table>
Figure 1.
Figure 2A.
Figure 2B. BTC single-plex

Figure 2C. TGF-α single-plex
Figure 3A.
Figure 3B.

Figure 3C.
Figure 4.
Figure 5A.
BTC

$y = 0.4112x + 0.1078$

$R^2 = 0.8385$

Figure 5B.
Figure 5C.
AR stability in serum

Figure 6A.
BTC stability in serum

Figure 6B.
Figure 6C.