Bio-Plex Pro™ TGF-β Assays for Simultaneous Profiling of TGF-β1, -β2, and -β3 in Human and Animal Models

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Introduction

Transforming growth factor-beta (TGF-β) belongs to a group of structurally related ligands widely expressed from *C. elegans* and *Drosophila* to mammals (Licona-Limon and Soldevila. 2007). The biological function of TGF-β is broad: it regulates cell replication, cell differentiation, bone formation, angiogenesis, hematopoiesis, cell cycle progression, and cellular migration. All these processes are important in tissue development, wound healing, and tumorigenesis (Chin et al. 2004, Branton and Kopp. 1999). The TGF-β subfamily consists of TGF-β1, -β2, and -β3. These isoforms arise from different genes and chromosomes and share extensive regions of similarity in their amino acids. TGF-β is found in essentially all tissue types, particularly in lung, kidney, bone, and placental tissues. The protein is secreted from many parenchymal cell types as a high molecular weight protein complex that is composed of three proteins: the mature TGF-β dimer, the latency-associated peptide (LAP), and the latent TGF-β binding protein (LTBP) (Chin et al. 2004). In many TGF-β assays, this protein complex must be acid-treated to release the mature, immunoreactive form of TGF-β.

Using the Luminex xMAP technology, we have developed a panel of magnetic bead–based multiplex immunoassays for measuring TGF-β1, -β2, and -β3 in human and animal models. The assays were validated with multiple matrices: serum, plasma, urine, and milk samples (human, mouse, and rat) and cell culture medium. Assay robustness was evaluated with respect to the following parameters: assay range (upper and lower limits of quantitation), sensitivity (limit of detection), intra- and inter-assay precision, specificity, dilution linearity, and parallelism. The validation demonstrated excellent performance in sensitivity, accuracy, and precision.

The Bio-Plex® Multiplex Array System

The Bio-Plex multiplex array system is built around three core technologies. The first is the family of fluorescently dyed bead particles to which biomolecules are bound. The second is a flow cytometer equipped with two lasers and associated optics to measure biochemical reactions that occur on the surfaces of the beads. The third is a high-speed digital signal processor that efficiently manages the fluorescent output. The system employs a patented multiplexing technology that uses up to 100 unique bead regions on magnetic polystyrene bead sets. Each set of beads is dyed with different ratios of two fluorophores (classification dyes CL1 and CL2).

Each of the classification dyes emits at a distinct wavelength, and both have significant absorption at 635 nm. The reporter is a third fluorophore (phycoerythrin-conjugated streptavidin, SA-PE) that absorbs maximally at 532 nm and emits at a third distinct wavelength. The detector consists of a flow cell designed such that the magnetic beads flow in single file by laminar flow through a region illuminated by two lasers. Upon excitation, the beads emit light at three wavelengths, two from the classification dyes and one from the reporter dye. The quantity of CL1 and CL2 light distinguishes one analyte-specific bead from another, while the emission from the reporter fluorophore is proportional to the quantity of analyte present in the sample.

The TGF-β assay employs a standard sandwich enzyme immunoassay method using a 96-well plate format. A capture antibody specifically directed against the analyte of interest is covalently attached to the bead particles. The antibody-coupled beads are allowed to react with a sample containing an unknown amount of analyte. After performing a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the analyte is added to the beads. The result is the formation of a sandwich of antibodies around the specific analyte. The reaction mixture is detected by the addition of the reporter SA-PE, which binds to the biotinylated detection antibodies. The constituents of each well are drawn up into the flow-based Bio-Plex suspension array system, which identifies and quantitates each specific reaction based on bead color and fluorescence intensity.

Procedure

The TGF-β assays were performed at ambient temperature using a 120 min (sample + beads) – 60 min (detection antibody) – 30 min (SA-PE) incubation protocol and a low photomultiplier tube (PMT) setting with the Bio-Plex reader. The automated Bio-Plex Pro wash station was used for all the washing steps. Data analysis was performed using the Bio-Plex Manager™ software version 6.0.

Sample Preparation

A wide range of samples, including serum, plasma, urine, milk, and cell culture medium can be analyzed with the TGF-β assays. To measure immunoreactive TGF-β, it is necessary to subject all sample types to an acid treatment procedure (1 volume of 1 N HCl to 5 volumes of sample), which is followed by a neutralization step (with a similar volume of 1.2 N NaOH/0.5 M HEPES). In this study, the neutralized serum and plasma samples were diluted to a final dilution of 1:16. Urine and milk samples were diluted 1:4 using a buffer that is similar to the sample matrix. The optimal dilution factor may vary depending on the samples, and further adjustment should be determined by the end user. In addition, some serum-containing culture media may contain high concentrations of TGF-β. Therefore, a control should be run in each assay to determine the baseline level of TGF-β in the medium.

Performance Characteristics

Five main assay parameters essential for method validation are assay working range, precision, sensitivity, accuracy, and specificity. Assay working range, also known as reportable range, is defined as an interval between the upper limit of quantification (ULOQ) and the lower limit of quantification (LLOQ) in which both intra-assay precision and standard curve accuracy are demonstrated. The TGF-β assays are designed to meet an intra-assay precision of ≤10% and a standard curve recovery of 80–120%. In this study, both TGF-β1 and TGF-β3 recorded greater than 4 logs in working assay range, and TGF-β2 recorded greater than 3 logs (Table 1). This hallmark feature differentiates the Bio-Plex assays from the 2 to 3 logs of dynamic range typically recorded on a conventional ELISA standard curve.

To evaluate the precision profile of the assays, intra-assay precision (within-run coefficient of variation; %CV) was calculated from three replicates at each standard dilution point obtained from a representative assay. Inter-assay precision (between-run %CV) was derived from three independent assays of a series of spiked controls prepared in a serum matrix. All three TGF-β isoforms recorded <10% in both intra- and inter-assay %CV (Table 1). Similar results were obtained with the singleplex configuration. Assay sensitivity, defined as the limit of detection (LOD), was calculated by adding two standard deviations to the mean background median fluorescence intensity (MFI) of the blank and calculating the corresponding concentration in pg/ml. The results showed that these assays are capable of measuring single-digit (pg/ml) levels for all three isoforms. This sensitivity is particularly crucial for TGF-β3, as the level of this isoform may not be easily measurable on ELISA or on competitors' assays on a similar platform.

Assay accuracy (recovery) was calculated as the percentage of the observed concentration value of a target antigen relative to the expected value. This parameter was investigated in both serum and RPMI cell culture medium by evaluating the recovery of the standard curve plus a series of spike controls prepared in serum matrix. The recovery data suggest that at least seven of the eight standard points are robust within the 80–120% recovery specification. This observation is supported by similar findings on the seven levels of spike controls (Table 2).

Table 2. Standard curve and spike control recovery.

	Standard Curve Recovery, %		
Standard Point	**TGF-β1**	**TGF-β2**	**TGF-β3**
S1	103	98	99
S2	99	102	101
S3	101	98	99
S4	101	109	102
S5	95	88	95
S6	108	111	111
S7	111	109	92
S8	41	83	105
	Spike Recovery, %		
Spike Control	**TGF-β1**	**TGF-β2**	**TGF-β3**
Spike 1	100	116	107
Spike 2	110	121	111
Spike 3	93	122	108
Spike 4	93	125	106
Spike 5	104	105	111
Spike 6	107	119	112
Spike 7	113	106	119

Table 1. Assay ranges (pg/ml) of 3-plex assays in serum matrix.

	Assay Working Ranges, pg/ml		Assay Precision		Assay Sensitivity, pg/ml
Analytes	**LLOQ**	**ULOQ**	**Intra-assay %CV**	**Inter-assay %CV**	**LOD**
TGF-β1	1.7	27,616	4.5	4.9	3.9
TGF-β2	14.7	30,080	6.3	9.1	1.9
TGF-β3	2.8	15,031	6.9	8.2	0.5

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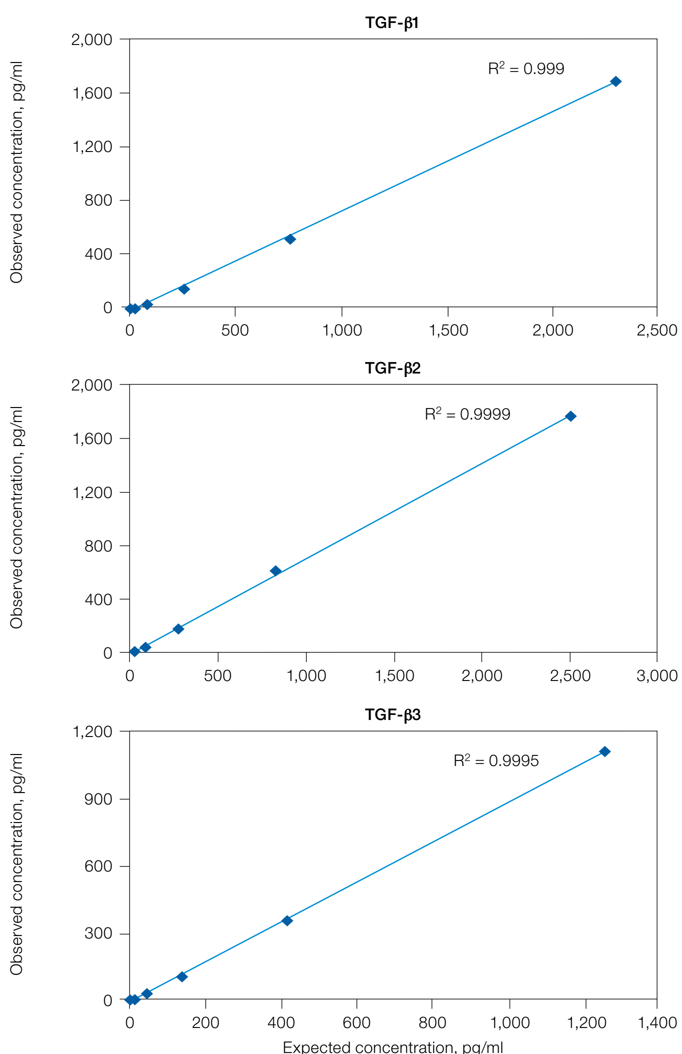
Assay specificity was examined by subjecting the assay to single-antigen and single-detection cross-reactivity studies. The single-antigen study evaluates the specificity of a capture antibody. This is conducted by testing an individual antigen in the presence of multiplexed capture beads and detection antibody. The single-detection study evaluates the specificity of the detection antibody. This is conducted by testing the individual detection antibody in the presence of multiplexed antigens and capture beads. These tests indicated <10% cross-reactivity among the three assays; thus, these assays can be multiplexed with ease.

Dilution linearity was investigated in these assays to ensure that analytes present in concentrations above the LLOQ can be diluted and measured accurately within the assay working range. Linearity of dilution was examined by diluting spiked samples with human serum or plasma in a 1:3 serial dilution. The observed and expected sample concentrations within the assay working range were plotted for each analyte. The correlation coefficient (R^2 value) generated by linear regression analysis reflects the linearity of dilution for that assay. In both sample matrices, the R^2 values are 0.990 or higher for all three assays (Figure 1). Similar results were obtained with the singleplex assays (data not shown).



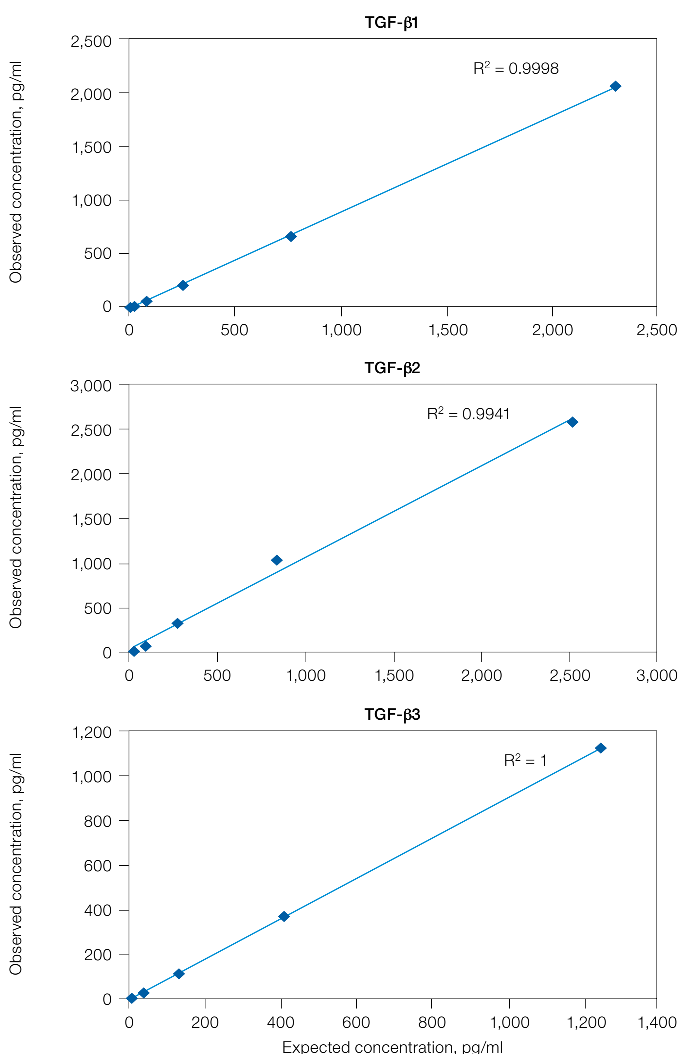


Fig. 1. Linearity of dilution plots for multiplex assays of TGF-β1, TGF-β2, and TGF-β3 in human serum and plasma.

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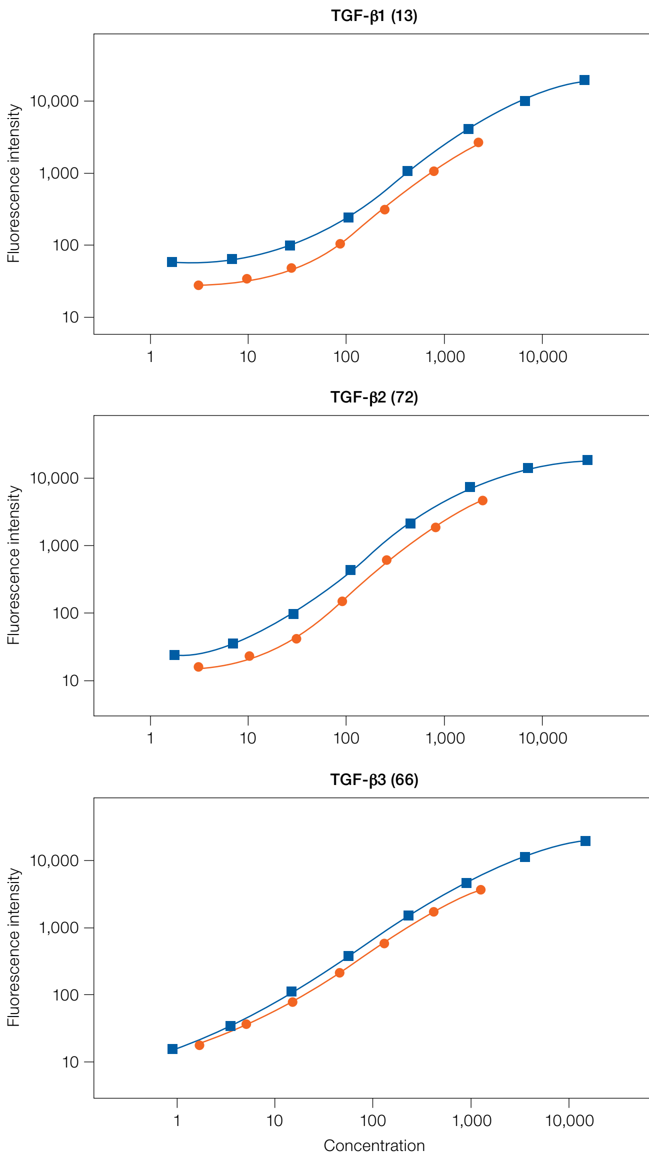
Parallelism analysis was also performed in serum and plasma matrices to ensure comparable binding characteristics between the reference standards and the samples. This was investigated by comparing the slope values of spike concentration response curves in human serum or plasma with that of a standard curve in standard diluent. Figure 2 shows that in both sample matrices, all three assays reasonably predict the relative quantity of an analyte by demonstrating a parallel dose response between the standard curve and the spike concentration response curve. Overall, the percentage difference in slope values was <20% for all three assays in both singleplex and multiplex configurations.



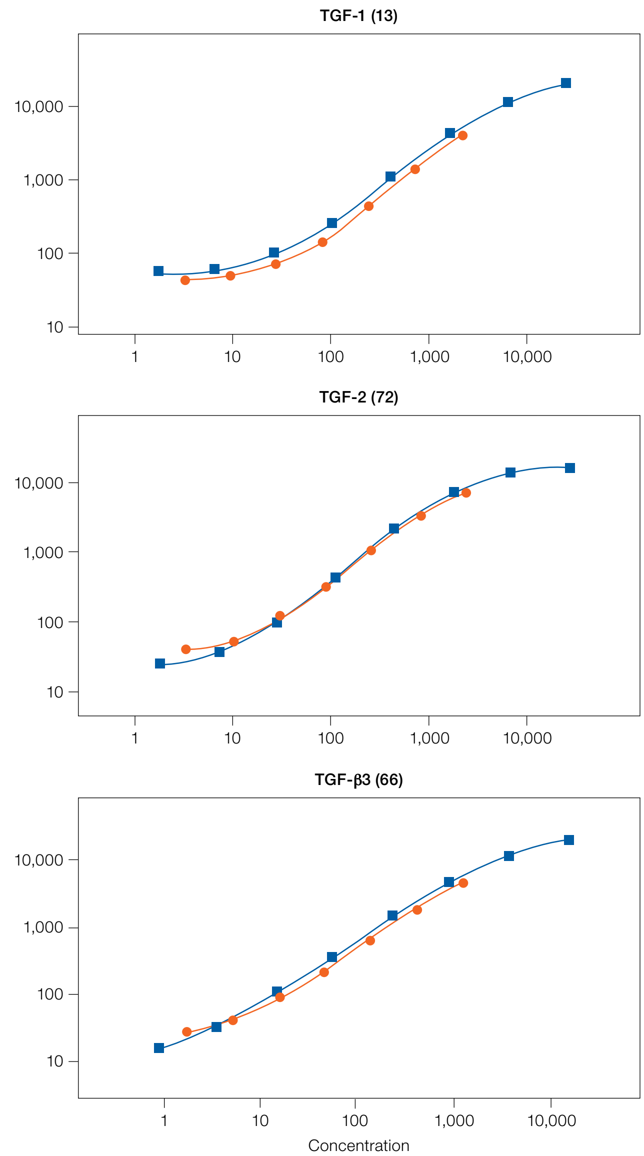


Fig. 2. Assay parallelism for multiplex assays of TGF-β1, TGF-β2, and TGF-β3 in human serum and plasma. The differences in the slopes of the standard curves and the corresponding spike concentration response curves in serum and plasma are <20% for all three assays in both singleplex and multiplex configurations. Spiked samples (●); standards (■).

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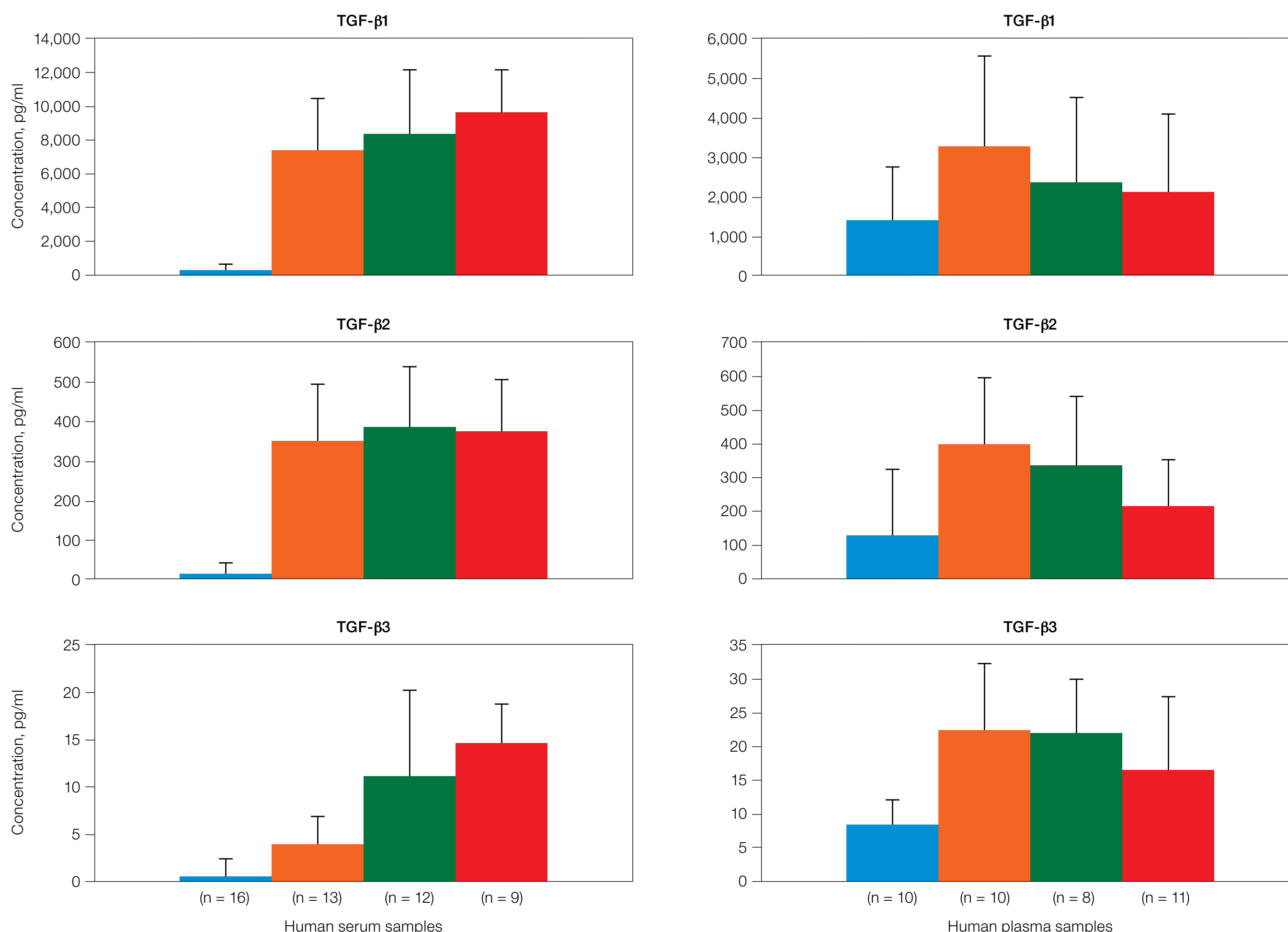

Fig. 3. Results of TGF-β assays on human serum and plasma samples from normal donors and from breast, colon, and lung cancer patients. In serum samples, TGF-β1 levels were significantly increased by 18-, 21-, and 24-fold, TGF-β2 levels were significantly increased by 19-, 21-, and 20-fold, and TGF-β3 levels were significantly elevated by 4-, 10-, and 13-fold, respectively, in breast, colon, and lung cancer patients compared with the levels in normal serum. In the plasma samples, TGF-β1 was significantly elevated by 2.3-fold in breast cancer patients, TGF-β2 levels were significantly increased by 3.1- and 2.7-fold in breast and colon cancer patients, and TGF-β3 levels were significantly increased by 2.3-, 2.3-, and 1.8-fold in breast, colon, and lung cancer patients, respectively, compared with the levels in normal plasma. All data were analyzed with Bio-Plex Manager 6.0. The bar represents the mean ± SD for each group of samples. Statistical analysis was performed using Student's *t*-test, and a *P*-value < 0.05 was considered significant. Serum and plasma samples were not from the same donors. Normal (■); breast cancer (■); colon cancer (■); lung cancer (■).

Validation with Biological Samples

To further evaluate assay robustness in key biological matrices, TGF-β assays were validated with human, rat, and mouse serum, plasma, milk, and urine samples. All three TGF-β isoforms were detected in these sample types from all species. Figure 3 presents the data for the human serum (left) and plasma (right) samples from normal donors and from breast, colon, and lung cancer patients. The levels of TGF-β1, -β2, and -β3 were found to be elevated to varying degrees in all three cancer groups relative to levels detected in a control group comprising healthy donors. The levels of these cytokines typically differ between serum and plasma matrices. Hence, it is crucial to conduct a study with only one type of matrix to ensure data continuity. Measurements that fall in the linear portion of the standard curve are likely to result in better precision and accuracy.

Comparison with other Luminex Platforms

To provide end users the flexibility of choosing other Luminex platforms, the TGF-β assays were evaluated on both the Luminex FLEXMAP 3D and MAGPIX systems. In all key sample matrices evaluated, the Bio-Plex 200 platform showed excellent agreement with both the FLEXMAP 3D and the MAGPIX systems in sample readout. The MFI values were comparable between Bio-Plex 200 and MAGPIX. The MFI values obtained from FLEXMAP 3D were higher due to its enhanced PMT setting. However, the sample concentrations determined with these three instruments were comparable (Figure 4).

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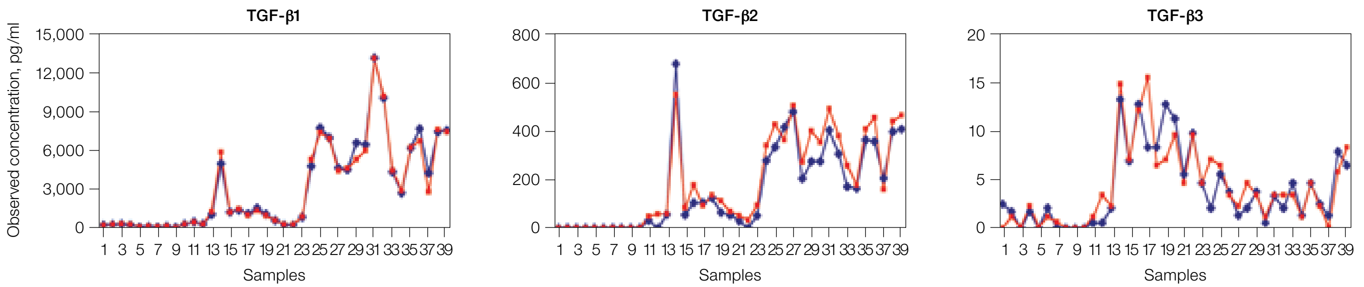
Bio-Plex 200 and MAGPIX



Bio-Plex 200 and FLEXMAP 3D

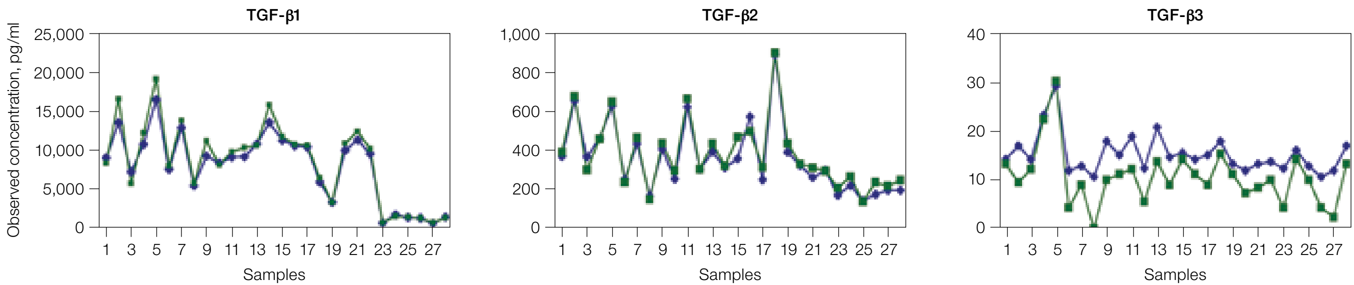


Fig. 4. Comparison of the Bio-Plex 200 with the MAGPIX and FLEXMAP 3D platforms. One plate of assayed serum and plasma samples was read with the Bio-Plex 200 followed by reading with the Luminex MAGPIX system. The sample concentrations obtained with the two instruments were similar. Another set of samples was read with the Bio-Plex 200 followed by reading with the FLEXMAP 3D system, and the obtained sample concentrations were again similar. Bio-Plex 200 (◆); MagPix (■); FlexMap 3D (■).

Conclusions

The TGF-β assays are specifically designed for quantifying TGF-β1, -β2, and -β3 in key sample matrices such as serum, plasma, urine, milk, and tissue culture medium. By utilizing magnetic beads and an automated plate washer, the assay panel can simultaneously measure three biomarkers in each sample well. The ability to multiplex significantly reduces cost, labor, and sampling volume when compared with more traditional methods such as ELISA. In addition, this assay panel is compatible with other Luminex readers such as the FLEXMAP 3D and MAGPIX systems.

These assays are able to detect all three TGF-β isoforms in serum, plasma, urine,and milk samples from human, mouse, and rat. Overall, when compared with competitive product lines, either ELISA or bead-based, the Bio-Plex Pro TGF-β assays showed similar or better performance.

The Bio-Plex Pro TGF-β assays are offered as a 3-plex premixed assay. This 1 x 96-well assay panel includes premixed coupled magnetic beads and detection antibodies, standards, assay buffer, wash buffer, detection antibody diluent, SA-PE, a filter plate, a flat bottom plate, sealing tape, standard diluent, sample diluent, and an instruction manual. Users also have the option to select from any of these three markers to tailor experiments specifically to their research studies.

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