Version 4.0

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BT-plex™ High Sensitivity Human Alzheimer's Disease Biomarker Panel

96-Well Plate Assay

User Manual

Catalog No. X202101A

The BT-plex™ High Sensitivity Human Alzheimer's Disease Biomarker Panel (5-plex) is a bead-based multiplex assay panel, using magnetic fluorescence-encoded beads for use with regular flow cytometers. The panel allows simultaneous quantification of Amyloid Beta 1-40 (Aßx40), Amyloid Beta 1-42 (Aßx42), Alpha-Synuclein (α-SYN), Total Tau, and Phosphorylated Tau at Threonine 181 site (pTau-181), or any combination of the 5 key targets, which are the major biomarkers for neurodegenerative diseases. The kit provides sensitive detection, ease of use, and is analytically robust in its assay performance characteristics. The multiplex biomarker assay panel allows comprehensive evaluation of neurodegenerative diseases particularly Alzheimer Disease (AD). Sample types tested include cerebral spinal fluid (CSF), serum, and plasma.

The BT-plex™ Human AD Biomarker Panel is designed to allow flexible customization. Users can select any combination of the 5 targets within the panel. Please visit www.biotimesinc.com for more product information or contact us (support@biotimesinc.com) to discuss your specific needs.

For Research Use Only

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Warning:

1. This reagent may contain Sodium Azide and other common laboratory chemicals. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion.
2. Biological samples should be handled as potentially hazardous. Follow universal precautions as established by the Center for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
3. Do not mix or substitute reagents from different kits or lots. Reagents from different manufacturers should never be used with this kit.
4. Do not use this kit beyond its expiration date.
5. SA-PE and fluorescent beads are light-sensitive. Minimize light exposure.
6. DO not freeze SA-PE and beads.

It is important that you read this entire manual carefully before starting your experiment.

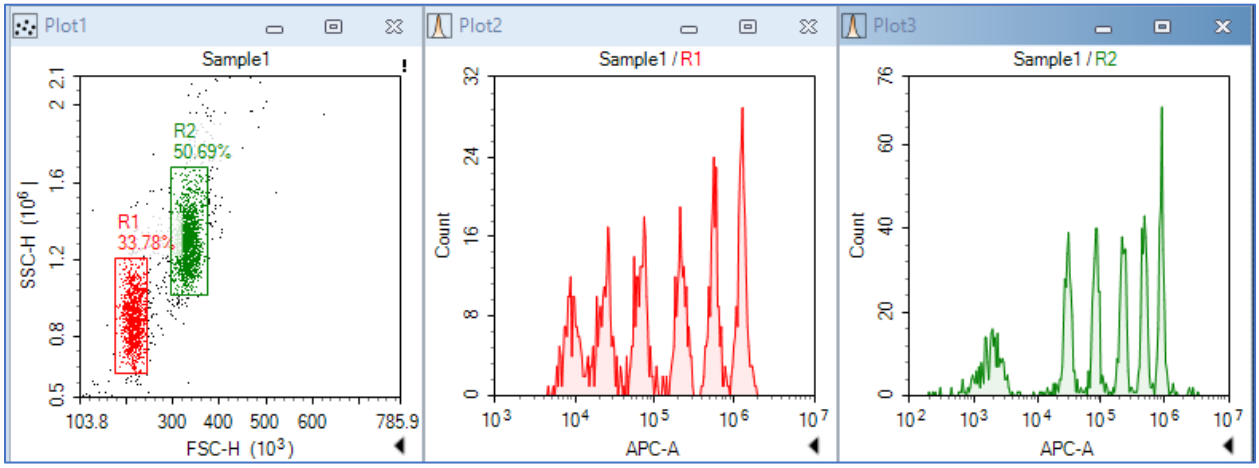
1. ASSAY PRINCIPLES

BT-plex™ bead-based kits use the same principle as typical sandwich immunoassays. Magnetic beads are differentiated by a combination of bead size and internal fluorescence intensities. Each bead set has a functional surface and is chemically conjugated with a specific capture molecule (e.g., antibody or binding protein) on the surface of the bead specific for a given analyte. When a selected group of beads with different Bead IDs are mixed and incubated with a sample, each target analyte in the sample will bind to its specific capture beads. A mixture of biotinylated detection antibodies specific to the targets are added, each detection antibody in the cocktail will bind to its specific analyte bound on the capture beads, thus forming capture bead-analyte-detection antibody sandwiches. Streptavidin-phycoerythrin (SA-PE) subsequently binds to the biotin group on the detection antibodies, providing a fluorescent signal with intensities in proportion to the quantity of bound analytes. With a flow cytometer reader, analyte-specific populations can be segregated and phycoerythrin fluorescent signal quantified. The concentration of a particular analyte is determined using a standard curve generated in the same assay.

2. BEAD

The BT-plex assays use two sizes of beads. Each size has several sub-populations that can be identified based on their forward scatter (FSC) and side scatter (SSC) profiles (Beads A, smaller beads and Beads B, larger beads). Each bead size can be further resolved based on their internal fluorescence intensities, which can be detected using FL4, or APC channel, depending on the type of flow cytometer used. The smaller Beads size A consists of 7 bead populations and the larger Bead size B consists of 6 bead populations. Using a combination of selected populations of A Beads and selected populations of B Beads, up to 13 targets can be simultaneously measured in a single sample using 1 multiplex assay panel. A 12-plex panel and associated bead populations are described in Figure 1.

Figure 1. Beads Differentiated by Size and Fluorescence Intensities*



*Gate R1 represent smaller beads (Size A) and Gate R2 represents larger beads (Size B). The R1 bead populations from lower to higher APC intensity are identified as A1, A2, A3, A4, A5, and A6, respectively. The R2 bead populations from lower to higher APC intensity are identified as B1, B2, B3, B4, B5, and B6, respectively.

For specific conjugated beads used in the Human AD Biomarker Panel 1, refer to Table 1 for exact assignment of Bead Identifications and top standard concentrations.

Table 1. Panel Targets, Bead ID, and Reference Standard Concentration

Target	Bead ID	Top Standard Conc.*
Human Aß1-40	A1	100 pg/mL
Human Aß1-42	A3	100 pg/mL
Human α-SYN	A5	100 ng/mL
Human pTau-181	B3	100 pg/mL
Human Total Tau	B6	5,000 pg/mL

Bead ID is used to associate bead populations with target analytes. For data analysis of FCS files, association of analyte and bead ID will be defined during the gating step of the data analysis software.

*The concentration of the target concentration when the lyophilized standard is reconstituted to 2.0 mL with Assay Buffer. The values assignments to standard subject to change from lot to lot.

3. STORAGE

All original kit components should be stored at between 2°C and 8°C. DO NOT FREEZE Pre-mixed Beads, Detection Antibodies or SA-PE.

Once the standard and Control Samples have been reconstituted, transfer contents to respective polypropylene microfuge vials. DO NOT STORE RECONSTITUTED STANDARD and Control Samples IN GLASS VIALS. Leftover reconstituted standard and Control Samples should be stored at ≤-70°C for future use. Avoid multiple freeze-thaw cycles. Discard any leftover diluted standards.

4. MATERIALS SUPPLIED

The BT-plex kit contains enough reagents for 100 tests with a small overfill to cover pipetting loss, listed in the table below. The reagent volume is sufficient for a 12-point standard curve with each point in duplicate and 72 individual sample wells.

Table 2: Kit Components information

Kit Components	Quantity	Volume	Item#
Human AD Panel 1 Pre-mixed Beads, Magnetic	1 Bottle	3.5 mL	X202101-01 or vary*
Human AD Panel 1 Detection Antibody	1 Bottle	3.5 mL	X202101-02
Human AD Panel 1 Standard, Lyophilized	1 Bottle	NA	X202101-03
BT-plex™ Streptavidin-Phycoerythrin Solution	1 Bottle	3.5 mL	X202101-04
BT-plex™ Assay Buffer	1 Bottle	25 mL	BTAB01
BT-plex™ 20X Wash Buffer	1 Bottle	25 mL	BTWB01
Low binding V-bottom plate**	1 Plate	NA	NA
Adhesive Plate sealer	2 sheets	NA	NA
Instruction Manual	1	NA	NA

*Catalog number of premixed beads vary with selection of targets. X202101-01 contains all 5 targets. Premixed beads are ready to use. **Low-binding V-bottom plate may be reused once after thorough cleaning.

For ordering a custom panel with individual beads, please use the table below to select target of interest and send the information alone with your contact details to sales@biotimesinc.com.

Target	Bead ID*	Target to select
Human Aß1-40	A1	
Human Aß1-42	A3	
Human α-SYN	A5	
Human pTau-181	B3	
Human Total Tau	B6	

5. MATERIALS TO BE PROVIDED BY THE USERS

5.1 A flow cytometer with two lasers (e.g., a 488 nm blue laser or 532 nm green laser and a 633-635 nm red laser) capable of distinguishing 575 nm and 660 nm is required. Partial list of compatible flow cytometers is tabulated below:

Flow Cytometer*	Classification Channel	Emission Wavelength	Reporter Channel	Emission Wavelength
Agilent Novocyte™	APC	660 nm	PE	572 nm
Beckman Coulter-CytoFLEX™	APC	660 nm	PE	585 nm
Beckman Gallios™	APC	660 nm	PE	575 nm
BD Accuri™ C6/ C6 Plus	APC, FL4	675 nm	PE, FL2	585 nm
BD FACSCalibur™, Canto™, Canto II, LSR™, LSRII™, LSR Fortessa™, FACSAria™, etc.	APC/FL4	660 nm	PE/FL2	575 nm
Other Flow Cytometers	Please refer to specific instrument instructions for best performing parameters			

5.2 Multichannel pipettes capable of dispensing 25 to 200 µL
5.3 Reagent reservoirs for multichannel pipetting
5.4 Polypropylene microfuge tubes (1.5 mL), mini-FACS tubes
5.5 Vortex mixer
5.6 Aluminum foil
5.7 Absorbent pads or paper towels
5.8 Orbital plate shaker (e.g., Lab-Line Instruments, model #4625)
5.9 Table-top centrifuges (e.g., Eppendorf centrifuge 5415 C) for microfuge tubes and samples
5.10 Magnetic separator for V Bottom Plate (e.g., Invitrogen A14179)
5.11 If filter plate is used, a vacuum filtration unit (e.g., Millipore MultiScreen ® HTS Vacuum Manifold, cat # MSVMHTS00 or equivalent) and a vacuum source.
5.12 If a magnetic 96-well separator is not available, a centrifuge with a swinging bucket adaptor for microtiter plates (e.g., Beckman Coulter Allegra™ 6R Centrifuge with MICROPLUS CARRIER adaptor for GH3.8 and JS4.3 Rotors)

6. ASSAY PREPARATION

6.1 Preparation of Serum Samples:

6.1.1 Allow the blood to clot for 30 minutes or longer and centrifuge for 10 minutes at 1,000 x g.

6.1.2 Remove serum and assay immediately or aliquot and store samples at ≤-20°C. Avoid multiple freeze/thaw cycles.

6.1.3 When using frozen samples, it is recommended that samples are thawed completely, mixed, and centrifuged to remove particulates prior to use.

6.2 Preparation of Plasma Samples:

6.2.1 Plasma collection using EDTA or citrate as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1,000 x g within 30 minutes after blood collection.

6.2.2 Remove plasma and assay immediately, or aliquot and store samples at ≤-20°C. Avoid multiple freeze/thaw cycles.

6.2.3 When using frozen samples, it is recommended that samples are thawed completely, mixed well, and centrifuged to remove particulates.

6.3 Preparation of Cerebrospinal fluid (CSF)

Lumbar puncture (spinal tap) is the most common method used clinically. Follow clinically acceptable practice in CSF sample collection and preparation.

6.4 Preparation of Tissue Culture Supernatant:

Centrifuge the sample to remove debris and assay immediately. If not possible, aliquot and store samples at ≤-20°C. Avoid multiple (>2) freeze/thaw cycles.

6.5 Preparation of Antibody-Immobilized Beads

- If pre-mixed beads are provided in the kit, vortex the bead bottle for 30 seconds prior to use.
- If individual beads are provided in the kit, the individual beads should be mixed with each other and diluted to 1X final concentration with Assay Buffer prior to use. To mix the diluted beads, follow the instructions below (a 3-plex panel is used as an example)
    - Vortex each bead vial for 30 seconds to completely resuspend the beads.
    - Calculate the amount of mixed and diluted beads needed for the assay. Prepare extra volume to compensate for pipetting loss.
    - Each reaction needs 50 µL of mixed and diluted beads. For 50 reactions, 3.0 mL of mixed beads is needed (2.5 mL plus 0.5 mL extra volume). For 96 reactions, prepare 5.5 mL of mixed beads.
    - To make 3.0 ml of 3-plex 1X diluted beads, transfer 0.25 mL of each of the 3 individual beads (12X concentrated) to a fresh tube (total bead volume = 0.75 mL) and add 2.25 mL of Assay Buffer to make the final volume of 3.0 mL.

6.6 Preparation of Wash Buffer

- Bring the 20X Wash Buffer to room temperature and mix to bring all salts into solution.
- Dilute 25 mL of 20X Wash Buffer with 475 mL deionized water. Store unused portions between 2°C and 8°C for up to one month.

6.7 Standard Preparation

6.7.1 Prior to use, reconstitute the lyophilized Human AD Panel 1 Standard Cocktail with 2.0 mL Assay Buffer.

6.7.2 Mix well and allow the vial to sit at room temperature for 10 minutes.

6.7.3 Transfer the reconstituted standard to an appropriately labeled polypropylene microfuge tube. This will be used as the top standard S11. Note: The top standard concentrations of analytes in this kit are currently set at the concentrations as described in Table 1. Top standard may subject to change from lot to lot.

6.7.4 Label 8 polypropylene microfuge tubes as S10, S9, S8, S7, S6, S5, S4, S3, S2 and S1, respectively.

6.7.5 Add 100 µL of Assay Buffer to each of the 11 tubes. Prepare 1:1 serial dilution of the top standard (S11) by transferring 100 µL of the top standard (S11) to the S10 tube and mix well. This will be the S10 standard.

6.7.6 In the same manner, perform serial 2-fold dilutions to obtain S9, S8, S7, S6, S5, S4, S3, S2 and S1 standards (see the table below using the top standard at 100 pg/mL as an example for Aßx40 and Aßx42).

6.7.7 Assay Buffer will be used as the 0 pg/mL standard (C0).

Standard Point	Serial Dilution	Assay Buffer to Add	Standard to Add	Standard Conc. for Aßx40, Aßx42, pTau-181 (pg/mL)	Standard Conc. for α-Synuclein (ng/mL)	Standard Conc. for Total Tau (pg/mL)
S11	NA	Add 2.0 mL	NA	100	100	5000
S10	1:2	100 µL	100 µL of S11	50.00	50.00	2,500.00
S9	1:4	100 µL	100 µL of S10	25.00	25.00	1,250.00
S8	1:8	100 µL	100 µL of S9	12.50	12.50	625.00
S7	1:16	100 µL	100 µL of S8	6.25	6.25	312.50
S6	1:32	100 µL	100 µL of S7	3.13	3.13	156.25
S5	1:64	100 µL	100 µL of S6	1.56	1.56	78.13
S4	1:128	100 µL	100 µL of S5	0.78	0.78	39.06
S3	1:256	100 µL	100 µL of S4	0.39	0.39	19.53
S2	1:512	100 µL	100 µL of S3	0.20	0.20	9.77
S1	1:1024	100 µL	100 µL of S2	0.10	0.10	4.88
S0	NA	100 µL	NA	0	0	0

A user may choose to make different serial dilutions starting from different top concentrations. In this case, reconstitution volume and dilution factors need to be adjusted accordingly.

6.8 Sample Dilution

6.8.1 Serum or plasma samples can be diluted 4-fold with Assay Buffer before being tested (e.g., dilute 25 µL of sample with 75 µL of Assay Buffer). Alternatively, 6.25 µL of neat serum samples can be added directly to plate wells followed by adding 18.75 µL of assay buffer.

6.8.2 CSF samples can be tested without dilutions.

6.8.3 For cell culture supernatant samples, the levels of targets vary greatly from sample to sample. A preliminary experiment may be needed to determine the appropriate dilution factor. If sample dilution is desired, dilution should be performed with corresponding fresh cell culture medium or Assay Buffer to ensure accurate measurement.

7. ASSAY PROCEDURE

The BT-plex™ assay can be performed in a low-binding V-bottom plate, U-bottom plate, a filter plate, or in small test tubes (e.g., microfuge tubes, mini-FACS tubes).

A low-binding V-Bottom 96-well plate is provided with each kit. The following assay protocols are described based on V-Bottom plate. For using small test tubes to run the assays, the protocols can be easily modified. Instead of using a magnetic plate separator, use centrifugation to separate the beads. For use with a filter plate (not provided) or for further modification of protocols, please contact support@biotimesinc.com to discuss recommendations.

Performing the Assay Using a V-, U-bottom Plate

- Allow all reagents to warm to room temperature (20-25°C) prior to use.
- Keep the plate upright during the entire procedures (except for quick decanting step) to avoid bead loss.
- The plate should be placed in the dark or wrapped with aluminum foil for all incubation steps.
- Standards and samples should ideally be run in duplicate and arranged on the plate and read on a flow cytometer in a consistent sequential order so that FCS file names and sample IDs are easily identified during data analysis.
- If an autosampler is used for plate reading, the reading orientation and sequential order should be carefully programed so that it matches the desired plate map.

7.1 Add **25 µL of diluted standards** to corresponding standard wells of the V-Bottom Plate, or a micro test tube.

7.2 Add 25 µL of samples (properly diluted) to sample wells. If sample pre-dilution is not desired, add 6.25 µL neat serum or plasma to each well, followed by adding 18.75 µL of Assay Buffer

7.3 Vortex mixed beads for 30 seconds. Add 25 µL of the Premixed beads to each well. (Note: During beads addition, shake mixed beads bottle intermittently to avoid bead settling).

7.4 Add 25 µL of Detection Antibody to each well. The total volume for each well should be 75 µL after Detection Antibody addition.

7.5 Seal the plate with a sheet of adhesive plate sealer. Cover the entire plate with aluminum foil to protect the plate from light.

7.6 Shake the plate at approximately 500-800 rpm on an orbital plate shaker for 5 min and then put the wrapped plate at 4℃ and incubate for approximately 18 h without having to use a plate shaker. Alternatively, the wrapped plate may be incubated for 2-4 hours at room temperature with moderate orbital shaking. Depending on type of shaker, the shaking speed should be high enough to keep beads in suspension during incubation, but not too high to cause spill-over. Overnight incubation provides better assay sensitivities.

7.7 Add 25 µL of SA-PE directly to each well containing the beads-standard/sample-detection antibody mixture. Seal the plate with the plate sealer. Wrap the entire plate with aluminum foil and shake the plate on a plate shaker for additional 30 minutes at room temperature.

7.8 Place the plate on a magnetic 96-well plate separator, wait for 1-2 min. Carefully remove liquid from the wells by decanting while holding the V-bottom plate on top of the magnetic 96-well separator, then blotting the plate on paper towels. Remove liquids as much as possible.

Note: If a magnetic 96-well plate separator is not available, centrifuge the plate at 250 x g for 5 minutes with a swinging bucket rotor. Immediately after centrifugation, decant the liquid into a container by quickly inverting and flicking the plate in one continuous and forceful motion. Blot the plate on a stack of clean paper towel and drain the remaining liquid from the well as much as possible.

7.9 Add 150 µL of 1X Wash Buffer to each well. Repeat Step 8.

7.10 Add 150 µL of 1x Wash Buffer. Shake the plate on the plate shaker for 1 minute.

7.11 Read samples on a flow cytometer. If the flow cytometer is equipped with an autosampler, the samples can be read directly from the plate. Please be sure to program the autosampler to resuspend beads in the well immediately before taking samples. If an autosampler is not available, the samples can be transferred from the plate to micro FACS (or FACS) tubes and read manually on the flow cytometer.

8. INSTRUMENT SETUP

Start up the instrument following the instrument manufacturer's recommendations.

8.1 Create a template for data acquisition using your instrument's data acquisition software. A template is a document or worksheet with density plots (e.g., FSC vs. SSC; PE vs. APC plots) that allows the user to perform machine setup and data acquisition.

8.2 Set up the PMT voltages of each channel, if necessary, to be used for data acquisition using the S0 and S7 wells of standards. Most modern flow cytometers have fixed PMT settings (e.g., Accuri C6™ and Novocyte™) and no PMT adjustment is needed.

8.3 Partial list of compatible flow cytometers:

Flow Cytometer*	Classification Channel	Emission Wavelength	Reporter Channel	Emission Wavelength
Agilent Novocyte™	APC	660 nm	PE	572 nm
Beckman Coulter-CytoFLEX™	APC	660 nm	PE	585 nm
Beckman Gallios™	APC	660 nm	PE	575 nm
BD Accuri™ C6/ C6 Plus	APC, FL4	675 nm	PE, FL2	585 nm
BD FACSCalibur™, Canto™, Canto II, LSR™, LSRII™, LSR Fortessa™, FACSAria™	APC/FL4	660 nm	PE/FL2	575 nm

9. DATA ACQUISITION AND ANALYSIS

9.1 Before reading samples, make sure that the flow cytometer is set up properly.

9.2 Create a new template or open an existing template (for details on how to create a cytometer-specific template, please refer to the Flow Cytometer Setup Guide).

9.3 Vortex each sample for 5 seconds before analysis for manual reading. Set plate agitation prior to each reading.

9.4 Set the flow rate to low. Set the number of gated beads to be acquired to about 150-200 per analyte (e.g., acquire 1,000-1,500 beads for a 5-plex assay). Instead of acquiring data using total events, create a large gate to include both Beads A and Beads B (gate A+B) and set to acquire the number of events in gate A + B. This will exclude majority of the debris from samples.

9.5 Read samples.

- If using an auto-sampler, read column by column (A1, B1, C1...A2, B2, C2...).
- When naming data files, try to use simple names with a consecutive numbering for easy data traceability and analysis.
- Store all FCS files in the same folder for each assay. If running multiple assays, create a separate folder for each assay.

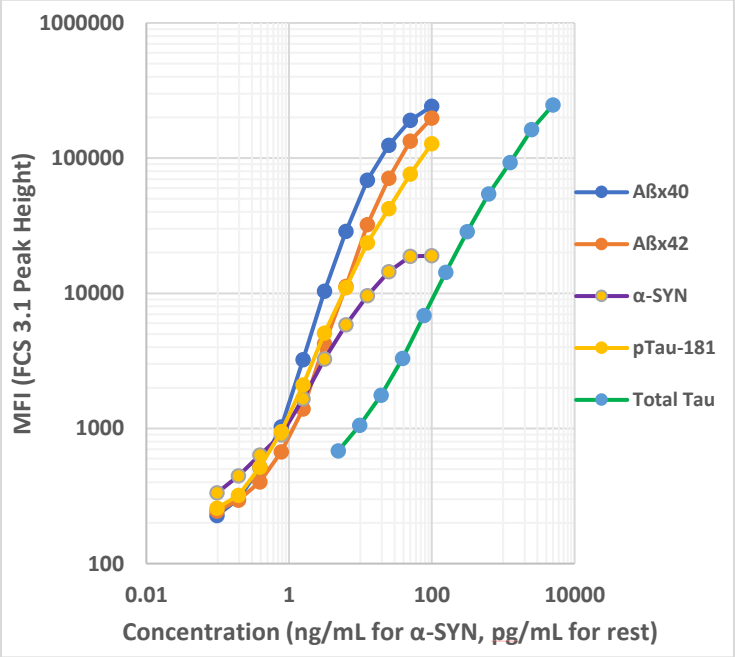
9.6 Proceed to data analysis using appropriate Data Analysis Software when data acquisition is completed.

- The FCS file generated on a flow cytometer should be analyzed using Vigenetech's multiplex data analysis software package for flow analysis. BT-plex Data Analysis Software will be available for downloading soon.
- FCS files may need to be exported for some flow cytometers. The data can be exported as FCS 3.0, FCS 3.1 format.
- BioTimes, Inc. offers free of charge data analysis for our customers. Customers can zip FCS files and send the data package to support@biotimesinc.com, or put the files

in a secure shared location such as Dropbox, along with detailed instructions on standard concentrations, plate map, and sample replicate information. Upon timely communication with us, we will analyze the data and send the results back within 3 business days.

10. ASSAY CHARACTERIZATION

10.1 Representative Standard Curves



This standard curve was generated using the BT-plex Human AD Panel 1 for demonstration purpose only. A standard curve must be run with each assay.

10.2 Assay Sensitivity (Pending final validation)

The assay sensitivity or minimum detectable concentration (MDC) is the theoretical limit of detection calculated using the Data Analysis Software by applying a 5-parameter curve fitting algorithm. The sensitivity data is for reference use only.

Target	MDC	Unit
Aßx40	**0.04**	**pg/mL**
Aßx42	**0.02**	**pg/mL**
α-SYN	**0.03**	**ng/mL**
pTau-181	**0.02**	**pg/mL**
Total Tau	**0.49**	**pg/mL**

10.3 Cross-Reactivity

There is no cross-reacting signal within the 5-plex assay panel. In addition, twenty-six different recombinant human proteins and complete bovine serum were tested using the BT-

plex High Sensitivity Human AD Biomarker Panel 1. There is no detectable cross-reacting signal.

10.4 Accuracy (Spike Recovery)

Samples with known concentrations of targets were spiked into 5 individual unknow serum samples at 3 levels of concentrations. The spiked samples were then quantified using Human AD Panel 1. The average % recovery is in general within acceptable ranges for all targets except for pTau-181, for which no data can be obtained reliably due to phosphorylation-related technical uncertainty in serum samples.

Target	Accuracy
Aß1-40	**99.0**
Aß1-42	**71.0**
α-SYN	**86.6**
pTau-181	**NA**
Total Tau	**87.4**

10.5 Sample Linearity of Dilution

Serum samples with high concentration of targets were diluted serially (2, 4, 8-fold) with Assay Buffer and then quantified using the High Sensitivity Human AD panel 1. On average, the linearity of sample dilution for all targets are acceptable.

Target	Linearity of Dilution
Aß1-40	**77.1**
Aß1-42	**72.5**
α-SYN	**93.0**
pTau-181	**104.5**
Total Tau	**86.9**

10.6 Intra-Assay Precision

Two independent samples were measured 8 times on the same plate. The overall intra-assay CV% is less than 10%.

Target	Intra-assay CV%
Aß1-40	**7.9**
Aß1-42	**6.6**
α-SYN	**3.0**
pTau-181	**5.1**
Total Tau	**3.1**

10.7 Inter-Assay Precision

Inter-assay CV is in general less than 15% for all assays within the panel.

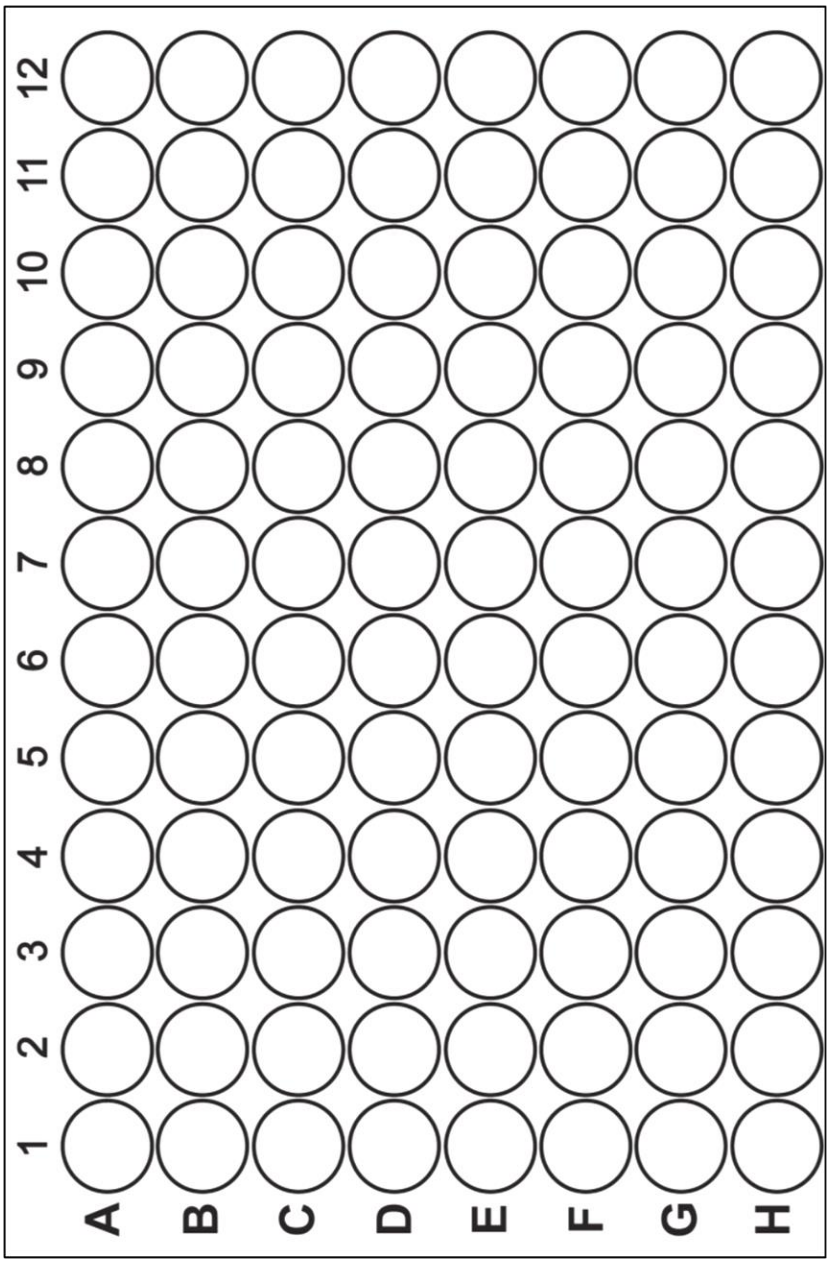
10.8 Biological Samples

Serum samples from 10 presumptive healthy donors were tested.

Target	Sample Detectability
Aß1-40	**Non-detectable to low pg/mL range**
Aß1-42	**Non-detectable to low pg/mL range**
α-SYN	**Mostly detectable at low ng/mL range**
pTau-181	**Mostly Non-detectable**
Total Tau	**Mostly Non-detectable**

Serum samples from recovered Covid-19 subjects demonstrated increased serum α-Synuclein concentrations post SARS-CoV-2 infection.

Limited number of commercially available CSF samples from clinically AD subjects were also evaluated and demonstrated signal changes consistent with literature.



BT-plex™ High Sensitivity Human Alzheimer's Disease Biomarker Panel is manufactured by BioTimes, Inc.,

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