ASSAY PROTOCOL

Bridge-It® L-Tryptophan Fluorescence Assay

(384-well microplate format)

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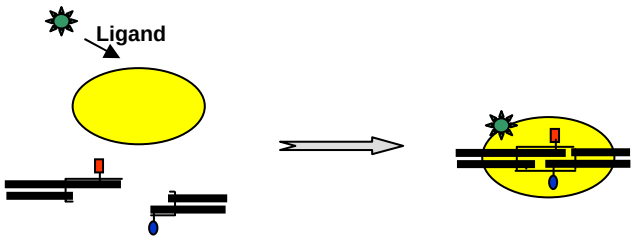
Introduction

**The Bridge-It®L-Tryptophan Fluorescence Assay Kit is intended solely for laboratory research and development (R&D) purposes. This product has not received government regulatory approval for use in diagnosis or treatment of diseases in humans or animals, monitoring of food products, or any applications other than laboratory R&D and it should not be utilized for such purposes.*

L-tryptophan (tryptophan) is one of eight essential amino acids that must be obtained from the diet. Tryptophan serves as a key building block for the synthesis of proteins and as a precursor for various brain neurotransmitters. Tryptophan is the only known precursor that can be converted into the neurotransmitter serotonin by the body. Serotonin promotes feelings of well being and calm and thereby helps to counterbalance the physiological affects of brain dopamine and the nor-adrenaline circuits that encourage fear, anger, tension, aggression, obsessive-compulsive activities, over-eating (especially of carbohydrates), migraine headache, depression and sleep disturbances. Melatonin, a metabolite of serotonin, is a sleep promoting natural hormone made by the pineal gland. In addition, tryptophan is a key precursor for niacin (vitamin B3), a vitamin that is essential for normal respiration, metabolism, and the synthesis of sex hormones. Because tryptophan plays such a critical role in the proper balancing of metabolism, mood and sleep patterns, insufficient dietary availability of this essential amino acid can lead to serious adverse clinical consequences.

Bridge-It® Fluorescence Assay Platform Design

The common property of all sequence-specific DNA binding proteins is their ability to bind with high affinity and specificity to a DNA duplex containing a unique nucleotide sequence - i.e., the DNA binding site for the protein. Mediomics' novel assay platform design relies on this common characteristic. A DNA duplex containing the sequence-specific DNA binding site for a given target protein is split into two DNA "half-site" duplexes each having a short single-stranded overhand. These single-stranded extensions are short enough so that in the absence of the target protein little spontaneous re-association occurs. When the target protein is present, however, its high affinity for the full-length DNA sequence will drive the re-association of the two half-site DNA duplexes. This re-association can be sensitively detected by incorporating appropriate fluorescence probes into each of the two DNA half-sites. The presence of the DNA binding protein is detected as an increase in the fluorescence signal. As shown schematically below, a simple variation of this basic platform design allows a DNA binding protein (yellow oval) to function as a sensitive biosensor for its specific ligand:



Bridge-It® L-Tryptophan Fluorescence Assay Principle

Eukaryotic cells contain an estimated 3,000 sequence-specific DNA binding proteins. These important proteins, acting either with or without a specific small molecule co-regulator (ligand), control all aspects of genomic DNA activity including gene expression, DNA replication, and DNA repair. Mediomics is applying its novel fluorescence assay platform to develop *in vitro* assays useful for rapidly and sensitively quantifying the activity of both DNA binding proteins and their small molecule ligands. L-tryptophan is a small molecule ligand that binds to and co-regulates the activity of the tryptophan repressor protein (also referred to as TrpR).

The Bridge-It® tryptophan fluorescence assay is based on the ability of TrpR, a bacterial DNA binding protein, to bind to its DNA binding site in an L-tryptophan dependent fashion. The two fluorochrome-labeled DNA half-fragments, each containing about one-half of the complete TrpR protein DNA binding site, are labeled one with fluorescein and the other with Oyster® 645 fluorophore. In the presence of L-tryptophan an increase in fluorescence signal can be detected as a result of the tryptophan dependent association of the two fluorophore-labeled DNA half-fragments. Tryptophan is readily detectable using the Bridge-It® tryptophan fluorescence assay in various types of test samples including bacterial growth medium, brain extract, yeast extract, as well as in human serum and urine. The linear range of the assay is 0.4 µM - 10 µM and the minimum tryptophan detection level is ~0.1 µM. The assay is highly specific for measuring L-tryptophan. This assay can be modified to become an assay for any enzyme reaction that uses L-Tryptophan either as a reactant or a product.

Bridge-It® L-Tryptophan Fluorescence Assay Reagents and Storage Requirements

The quantity of reagents contained in the assay kit is adequate to perform the indicated number of individual tryptophan measurements using a black 384-well non-binding surface microplate. Store the Bridge-It® L-Tryptophan Fluorescence Assay Kit in the freezer at -20ºC until needed for use. **Thaw only the appropriate number of reagent tubes needed for the planned experiment.** Each tube of Tryptophan Assay Solution A contains reagent adequate for performing fifty (50) tryptophan measurements using a 384-well black microplate. The tryptophan assay kit reagents will retain their activity for at least 2 months when stored frozen at –20°C. After thawing, any unused reagents may be stored tightly capped in the refrigerator at ~ 4°C for up to one week.

Tube Cap Color	Assay Reagent	ml/tube	Storage Conditions
Green	1 mM L-tryptophan standard in H_20	0.25	Once thawed, store refrigerated at 4ºC. **DO NOT RE-FREEZE.**
Violet	L-Tryptophan Assay Solution A	1.0	Once thawed, store refrigerated at 4ºC. Stable for at least 1-week. **DO NOT RE-FREEZE.**
Blue	Buffer W*	1.0	Once thawed, store refrigerated at 4ºC**.**

***Note:**
Buffer W may be used for diluting L-tryptophan standards and test samples.
384-well round bottom low volume non-binding surface black polystyrene microplates are recommended and may be purchased as individual plates from Mediomics, LLC (Catalog # 163301).

Bridge-It® L-Tryptophan Fluorescence Assay: General Notes

The following precautionary steps are recommended in order to optimize assay performance and reproducibility:

- Pipette slowly to avoid bubbling and to allow complete recovery and transfer of assay solutions.

- If more than one tube of L-Tryptophan Assay Solution A is required for experimental analysis, mix all Assay Solution A tubes together to ensure reagent consistency before use. Thaw only the number of tubes needed for the experiment.

- After the samples have been transferred into the wells of the 384-well black microplate, gently tap the microplate on the counter top to release any trapped bubbles. Although bubbles generally break with time, large bubbles may be broken with the tip of a small gauge needle (e.g., 27 gauge) being careful to avoid loss of volume through capillary action.

- Fluorescence readings can be affected by temperature. It is recommended for consistency of assay results that all microplates used in a given study be incubated as close to the same temperature as possible (preferably ~25°C).

- To ensure consistency of results, it is recommended that a volume of 18 µl of standard or sample be transferred into the wells of a 384-well black microplate.

- High salt concentration will affect the annealing of DNA in the assay. Thus, use of high salt concentrations should be avoided in preparing the test samples and tryptophan standards to be measured using the Bridge-It® L-tryptophan fluorescence assay.

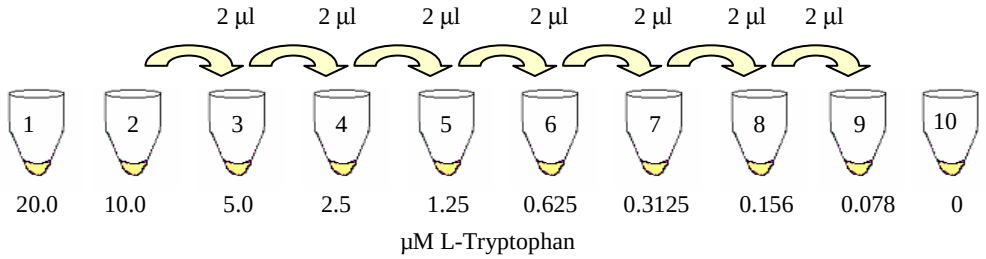
- Certain test samples (e.g., urine containing high levels of vitamins) may exhibit high background fluorescence. In order to take background fluorescence into account, it is recommended that the test sample be diluted in buffer alone (i.e., no assay solution included) and measured against a buffer blank (settings: excitation ~ 485 nm; emission: ~665 nm). Additional information concerning control of background fluorescence is presented elsewhere in this protocol (see data analysis, page 10).

Bridge-It® L-Tryptophan Assay Standards

If more than one tube of L-Tryptophan Assay Solution A is required for experimental analysis, mix all tubes together to ensure reagent consistency before use. Thaw only the needed number of Assay Solution A tubes needed for the experiment.

It is recommended that the L-tryptophan standard curve be prepared in Buffer W or under similar conditions of the test samples. If a buffer other than Assay Buffer W is used, a test to determine the effect of the alternate buffer on assay performance should be conducted prior to using it in the assay. The L-tryptophan standards should be prepared as follows:

1. Dilute the 1 mM L-tryptophan standard (green cap tube) 1:5 using Buffer W (blue cap tube) for a final concentration of 200 µM L-tryptophan. Prepare 10 polypropylene Eppendorf tubes. Add 2 µl of Buffer W to tubes 2-10. Add 2 µl of the 200 µM tryptophan stock to tubes 1 and 2. Mix the contents of tube 2 by pipetting up and down 5 times. Transfer 2 µl from tube 2 into tube 3. Continue serially diluting the tryptophan standard through tube 9. After mixing, discard 2 µl from tube 9. All tubes should contain a final volume of 2 µl of tryptophan standard solution.



Note: 20 µM is the final concentration of tube 1 when a total volume of 20 µl is used. It is equivalent to 400 pmol/well. The L-tryptophan standards may be diluted directly in the microtiter plate. Mix by slowly pipetting solution up and down 4-5 times (avoid bubbles).

2. Add 18 µl of L-Tryptophan Assay Solution A to each Eppendorf tube and vortex immediately for ~1 second at medium speed.

3. Carefully transfer 18 µl into the appropriate well of a 384-well black microplate. The transfer of 18 µl/well rather than 20 µl/well when assayed directly in the microplate will not affect assay sensitivity. It is recommended that the volume used for the standards and test samples be the same.

4. Cover the microplate with tinfoil to avoid evaporation and exposure to light. Incubate the microplate at room temperature (~ 25°C) for at least 30 minutes.

5. Read fluorescence signal intensity using a fluorescence microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm).

Determination of L-Tryptophan Levels in Various Samples

It is possible to use this assay to determine L-Tryptophan levels in various types of test samples including biological fluids and extracts of tissues and cells. Liquid samples (including standards) should be diluted in Buffer W. If a different buffer is used, a test to determine the effect of the different buffer on the assay performance should be conducted before using the assay. If the sample has an innate fluorescence, it is recommended a sample blank be measured at excitation ~485 nm; and emission ~665 nm (see page 10).

If more than one tube of L-Tryptophan Assay Solution A is required for experimental analysis, mix all of the Assay Solution A tubes together to ensure reagent consistency before use. Thaw only the number of tubes needed for the experiment.

Direct Analysis of a Liquid Sample:

1. Clarify the sample by centrifugation if necessary. Prepare several dilutions of the sample with Buffer W to ensure that the read-out falls within the linear range of the standard curve. Add 2 µl of sample to a polypropylene Eppendorf tube.

2. Add 18 µl of L-Tryptophan Assay Solution A to each Eppendorf tube and vortex immediately for ~1 second at medium speed.

3. Carefully transfer 18 µl from each Eppendorf tube into the appropriate well of a 384-well black non-binding surface microplate.

4. Cover the microplate with tinfoil to avoid evaporation and exposure to light. Incubate the microplate at room temperature (~ 25°C) for at least 30 minutes.

5. Read fluorescence signal intensity using a fluorescence microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm).

6. **NOTE:** It is possible to use the assay to measure up to 20% v/v of liquid sample to be added directly to the Assay Solution A with only a small affect on assay performance. To do this, add 4 µl of the liquid test sample to 16 µl Tryptophan Assay Solution A. The standard curve including the blank should be prepared as previously described, but an additional 2 µl of Buffer W should be added to the 2 µl of standard in order to bring it to the same 4 µl volume used for the test samples. Add 16 µl of Tryptophan Assay Solution A to each well and follow steps 4 and 5 above.

Increase Concentration of L-tryptophan Contained in Test Samples

If the L-tryptophan level contained in the sample extracts is found to be less than the lower limit of detection for the assay, the L-tryptophan in the sample extracts may be concentrated using lyophilization. If the protein content present in such concentrated samples is found to be problematic, ethanol extraction of the sample extract followed by centrifugation and lyophilization of the supernatant (i.e., ethanol containing L-trytophan) is recommended. High salt concentrations affect DNA annealing and should be taken into consideration. It is not recommended that the sample extracts be lyophilized unless they are contained in a volatile solvent such as ethanol. Samples containing very low levels of L-tryptophan may be concentrated for use in the assay as follows:

1. Dilute the test samples with cold absolute (100%) ethanol to achieve a final concentration of 70% ethanol. Tightly cap all tubes, vortex and incubate on ice for 15 minutes.

2. Following the 15 minute incubation on ice, centrifuge the sample at ~12,000g for 10 minutes at 4˚C.

3. Carefully transfer the supernatants into a fresh labeled polypropylene Eppendorf tube and lyophilize the supernatant samples (e.g., speed vac dry). Lyophilized samples that are not to be assayed for L-tryptophan content immediately should be stored at -20˚C until needed for analysis.

4. It is recommended that tubes containing the 10 assay standards (2 µl per tube) be prepared as described on page 7, lyophilized and stored at -20˚C for assay with the lyophilized test samples.

5. Add 20 µl of Tryptophan Assay Solution A to each of the lyophilized standards and test samples. Vortex for ~1 second at medium speed.

6. Transfer 18 µl into the appropriate well of a 384-well black non-binding surface microplate.

7. Cover the microplate with tinfoil to minimize evaporation and exposure to light. Incubate the covered microplate at room temperature (~ 25°C) for at least 30 minutes.

8. Read fluorescence signal intensity using a fluorescence microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm).

If the tryptophan content is too high for measurement using direct dissolution in Trytophan Assay Solution A after ethanol extraction and lyophilization, the samples may be diluted in Buffer W or in water (depending on salt concentration) for direct measurement as described above.

Bridge-It® L-Tryptophan Fluorescence Assay: Data Analysis

There are several ways of analyzing the fluorescent signal intensity readouts obtained using this assay.

1. Raw fluorescence intensity may be used as a signal proportional to L-tryptophan concentration (the x-axis is common log; the y-axis is linear).

2. Relative fluorescence (RF) change may be calculated from the raw fluorescence values. RF values are highly reproducible for the same L-tryptophan concentrations and do not depend significantly on the particular microplate reader instrument that is used to read the fluorescence signals.

   RF = Relative Fluorescence
   F_0 = fluorescent intensity of the 0 L-tryptophan standard (see page 7)
   F = fluorescence of L-tryptophan standard or test sample
   Fb = fluorescence of buffer blank (Buffer W only)
   Fbg = native sample fluorescence

   - Relative fluorescence change (RF) may be calculated from the raw fluorescence values using the formula RF = $(F-F_0)/ F_0$, or alternatively:

   - Buffer and/or standard blanks may be used for the determination of a Buffer or Standard Adjusted Relative Fluorescence (Buffer Adjusted F-Fo/Fo).

   ➢ 18 µl of Buffer W (Fb) is added per each of 2 wells per microtitre plate in the experiment. Read for fluorescence at the same time and conditions as the standard curves and samples. The average of the two buffer blanks is subtracted from the fluorescence readings (F) of both the standards and samples giving a "buffer adjusted" F-Fo/Fo" or:

   (F-Fb)- (Fo-Fb) / (Fo-Fb).

   ➢ Background fluorescence present in test samples (Fbg) may be accounted for by determining the fluorescence signal of the test sample versus that of Buffer W blank (Fb) (i.e., the fluorescence of test sample in the absence of labeled probes).

   The sample (or sample dilution) should be handled in the same manner as the sample that is actually being assayed. For example, add 2 µl of sample to 18 µl of Buffer W (Fbg), mix, and transfer 18 µl of the solution into the well of a 384-well microplate. Read the fluorescence of the samples compared to the fluorescence readings of the buffer blank (Fb) using a microplate reader (settings: excitation ~ 485 nm; emission ~ 665 nm). If Fbg is greater than Fb,

subtract the Fbg to eliminate the background sample fluorescence. The Relative Fluorescence (RF) then becomes

$$RF = [(F-Fbg) – (Fo-Fb)] / Fo-Fb).$$

3. Calculation of the final concentration of L-tryptophan in the test sample should incorporate the dilution factor. For example, when 2 µl of the sample is used in the assay and the concentration is found to be 5 µM, the concentration of test sample is 50 µM.

4. A “Sigma Plot” software program was used to analyze and graph the results obtained from the Bridge-It® Tryptophan Fluorescence Assay. Data were converted to RF values and plotted as a sigmoidal, 4-parameter graph (x-axis as common log, y-axis as linear).

Bridge-It® L-Tryptophan Fluorescence Assay Performance

L-Tryptophan Standard Curves

The following L-tryptophan standard curve was prepared using the Bridge-It® Tryptophan assay. Figure 1 shows the standard curve prepared in Buffer W with the fluorescence signal read after 30 minutes, 1.5, 3 and 5.5 hours following the addition of L-tryptophan Assay Solution A into the wells of the 384-well black microplate. The standard errors were from duplicate measurements. Figure 2 shows a portion of the same curve (0.07-1.25 µM L-tryptophan) at 30 minutes plotted to show the lower range of sensitivity. See data analysis on page 10 for additional information.

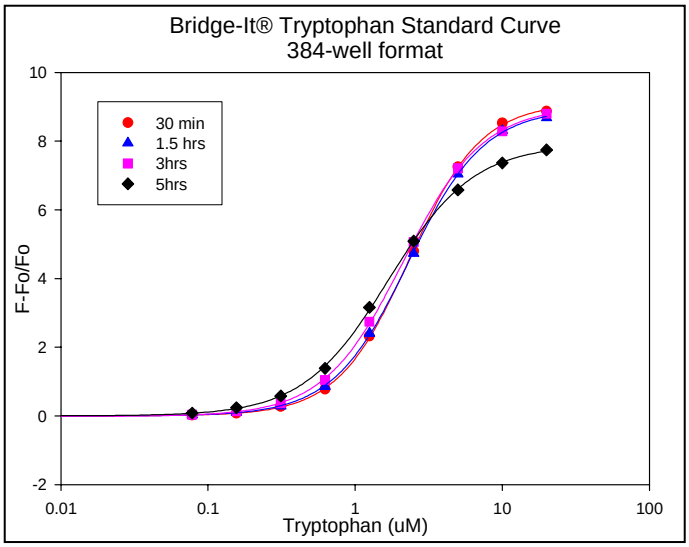


Figure 1

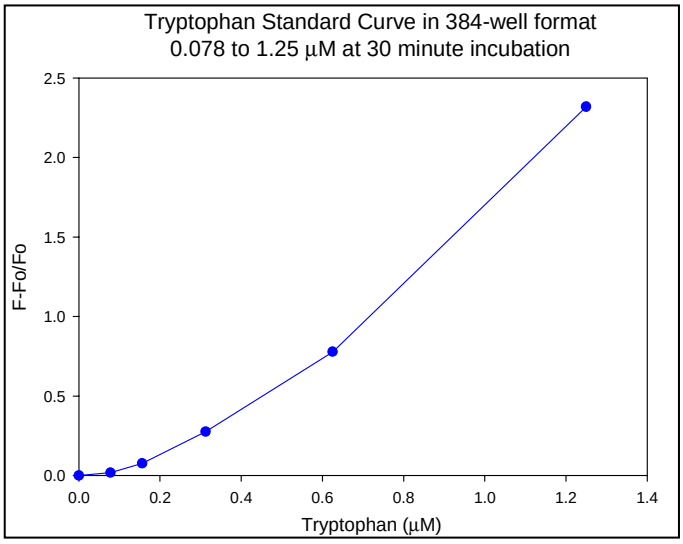


Figure 2

L-Tryptophan Uptake by Bacteria Growing in LB Broth

E. coli strain, DH5α, was seeded into LB Broth and grown overnight at 37°C. The following day, the culture was diluted to OD_{600nm} = 0.1. Cells were divided into two flasks, and incubated at 37°C with shaking. Samples were taken each hour for a period of up to 7 hours. Bacterial growth in the duplicate cultures was measured at OD_{600nm} at each time point.

Hourly samples were also taken and centrifuged at 12,000 rpm at 4°C for 10 minutes. The supernatants were aliquoted and immediately frozen. The frozen samples were subsequently thawed, diluted in Assay Buffer W, and analyzed for L-tryptophan content using both the Bridge-It® L-tryptophan fluorescence assay method and the AccQ-Tag HPLC procedure. Using the Bridge-It® L-Tryptophan Fluorescence Assay, LB Broth was found to contain 331.7 µM L-tryptophan at the beginning of the culture. The initial concentration of tryptophan is considered to be 100%. The results of these studies are presented in Figure 3.

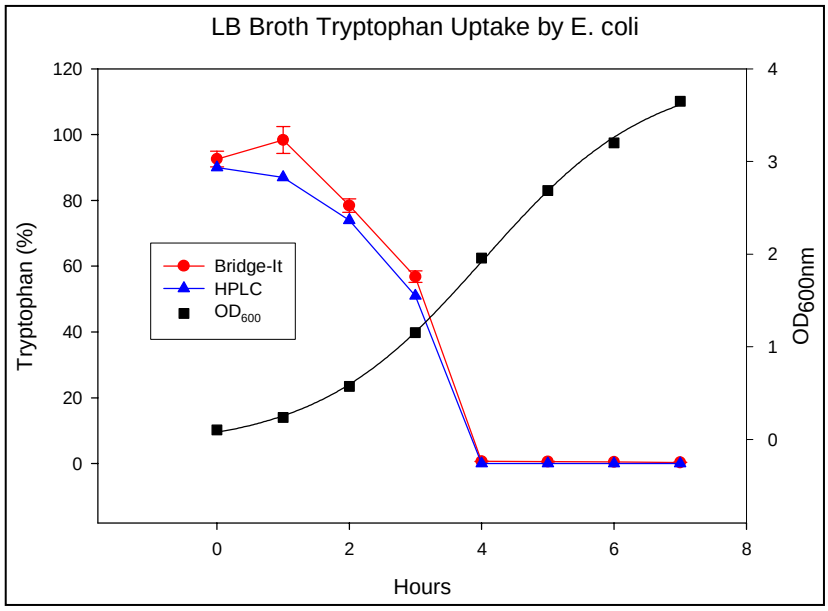


Figure 3

Determination of L-Tryptophan Levels in Biological Samples

Rat Brain Extract:
Rat brain was extracted using the following procedure. Two volumes per gram (v/g) of brain of 50mM NH_4HCO_3 were used to homogenize the rat brain tissue. After centrifugation at 12,000 rpm for 30 minutes at 4°C, the supernatant brain extract was transferred into another centrifuge tube and diluted with cold absolute (100%) ethanol to a final concentration of 70%. The tube was tightly capped and placed into an ice bath. After 30 minutes of incubation on ice, the ethanol-treated extract was clarified by centrifugation. The supernatant solution was aliquoted into 1 ml samples in Eppendorf tubes, lyophilized (speed vac dried) and stored frozen at -20ºC until needed for analysis. An aliquot was later resuspended in 80 µl of cold Buffer W and centrifuged at 12,000 rpm for 15 min at 4ºC. Results of the analysis of the rat brain extract for the presence of L-tryptophan are shown on Table 1. The L-tryptophan level in the rat brain extract that was prepared and analyzed in our laboratory was determined to be 5.6 nmol L-tryptophan per gram of wet weight rat brain tissue.

Yeast Cell Extract:
Yeast strain BY4743 was grown in Yeast-Bactopeptone-Dextrose (YBD) broth overnight at 30ºC with gentle agitation. The following day, the culture was diluted to a density of approximately 0.02 OD_{600nm} and again cultured overnight at 30ºC with gentle agitation. The yeast cells were harvested at a density of 8.0 OD_{600nm} by centrifugation at 4ºC. The yeast cells were washed 2-times with cold PBS and the cells pellet was resuspended in ~1/30th of the original volume of the culture using cold double distilled water. An equal volume of washed glass beads (0.5 mm) was added to the suspended cell pellet and vortexed at top speed for one minute followed by incubation on ice for one minute. This cycle of vortexing and incubating on ice was repeated 6-times. The sample was centrifuged and the supernatant yeast cell extract was diluted using absolute (100%) ethanol to a final concentration of 70% ethanol and incubated on ice for 10 minutes. A final centrifugation removed the resulting precipitate. The clarified supernatant containing the ethanol extracted L-tryptophan was divided into 1 ml aliquots, dried in a speed vac, and immediately frozen for storage in a freezer at -20ºC. The following day, a frozen aliquot was thawed, reconstituted using 30 µl cold autoclaved double distilled water, diluted 1:8 in Buffer W and centrifuged at 4ºC to remove any precipitation prior to performing the tryptophan analysis using the Bridge-It® L-tryptophan fluorescence assay. The results of this analysis are presented in Table 1. The tryptophan level in the yeast cells was determined to be 63.18 nmole L-tryptophan in 1x10^9 yeast cells.

Human Urine:
Samples of urine from two normal adult human subjects were centrifuged and the clarified urine (supernatant) was aliquoted (1 ml/tube) into clean labeled Eppendorf tubes and immediately frozen for storage in the freezer at -20ºC until needed for testing. Subsequently the frozen urine samples were thawed, diluted in cold Buffer W, and centrifuged again to remove any precipitate. The clarified supernatant urine was analyzed for L-tryptophan content using the Bridge-It® L-Tryptophan Fluorescence Assay. Urine dilutions of 1:4 or less were found to have a significant level of background fluorescence.

This background fluorescence was taken into account as part of the assay data analysis. The L-tryptophan levels found in two normal human urine specimens using the Bridge-It® L-Tryptophan Fluorescence Assay are presented in Table 1.

Human Blood:
Commercially-available, normal, pooled human serum and pooled plasma and also fresh human serum and plasma from a single normal donor were simultaneously analyzed for L-tryptophan using the Bridge-It® L-Tryptophan Fluorescence Assay. Following dilution in cold Buffer W, the serum and plasma samples were centrifuged to remove any precipitate prior to L-tryptophan analysis. The L-tryptophan levels in fasting normal human plasma and serum samples have been reported in the literature to be in the range of 24-79 µM for children (1-16 years old) and 20-95 µM for adults[5]. As shown in Table 1, the levels of L-tryptophan obtained for the samples of normal human serum and plasma using the Bridge-It® L-Tryptophan Fluorescence Assay fell within the reported normal range for L-tryptophan in normal human blood samples.

Table 1

L-Tryptophan Levels Found In Various Types of Test Samples.

Sample	Bridge-It® L-Tryptophan (µM)
Rat Brain Extract	10.5 ± 1.8
Yeast Cell Extract	234 ± 28
Human Urine 1	23.7 ± 2.6
Human Urine 2	19.5 ± 0.9
Pooled Human Serum	35.9 ± 0.7
Pooled Human Plasma	25.9 ± 6.1
Fresh Human Serum	20.6 ± 4.5
Fresh Human Plasma	23.1 ± 4.9

Note: The data represents the average value ± standard deviation (n = 4 to 7)

Specificity of the Bridge-It® L-Tryptophan Fluorescence Assay

To assess the specificity of the Bridge-It® L-Tryptophan Fluorescence Assay, all 20 amino acids were tested up to 100 µM concentration. Nineteen (19) amino acids (excluding L-tryptophan), were shown to have no cross-reactivity with L-tryptophan when they were measured using the Bridge-It® L-Tryptophan Fluorescence Assay. Further, D-tryptophan (minimum of 98% pure as measured by thin layer chromatography), serotonin, and 5'-HTP (a precursor of L-tryptophan) were also analyzed up to 20 µM concentration using the Bridge-It® L-Tryptophan Fluorescence Assay with no significant cross-reactivity observed as demonstrated by the results shown below in Figure 4.

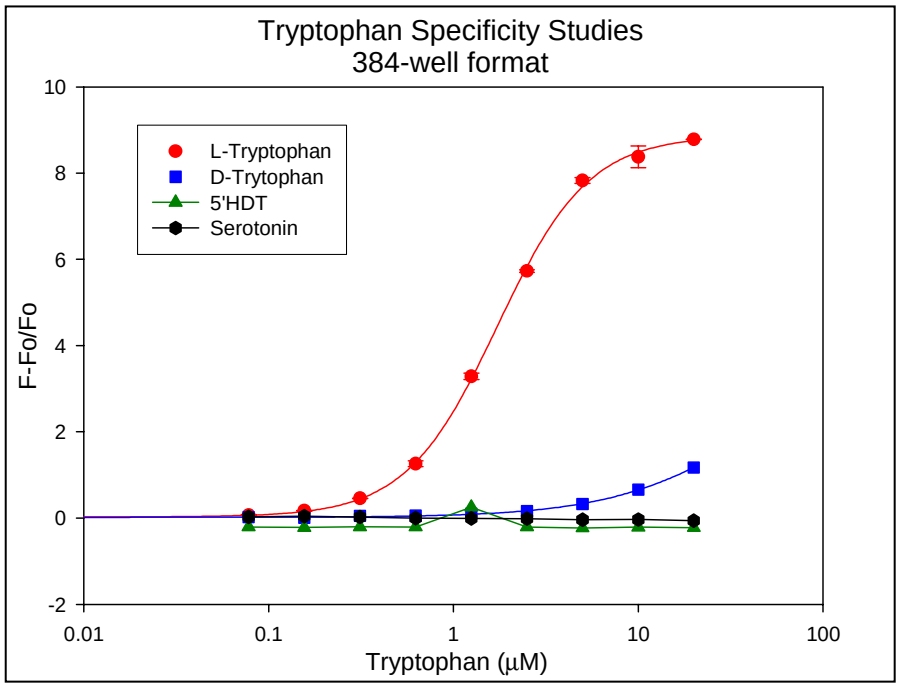


Figure 4

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3. US Patent No. 6,544,746, Rapid Proximity-Based Assay for the Detection and Quantification of DNA-Binding Proteins.
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Product Ordering Information

Bridge-It® L-Tryptophan Fluorescence Assay Kit (384-well microplate format):

Product Description[1,2]	No. Measurements	Catalog No.	Price[3]
Bridge-It® L-Tryptophan Fluorescence Assay Kit	100	1-1-1002A	$200.00
Bridge-It® L-Tryptophan Fluorescence Assay Kit	384	1-1-1002B	$765.00

384-well black microplate:

Product Description[1]	Catalog No.	Price[3]
384-well round-bottom low volume, non-binding surface, black polystyrene microplate	163301	$9.50/microplate

[1] Bridge-It® is a registered trademark of Mediomics, LLC, St. Louis, Missouri, USA.
[2] Oyster® is a registered trademark of Denovo Biolabels, GmbH, Munster, Germany.
[3] All prices are denominated in U.S. dollars. Shipping and handling cost will be applied. Prices shown may be changed without notice.

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