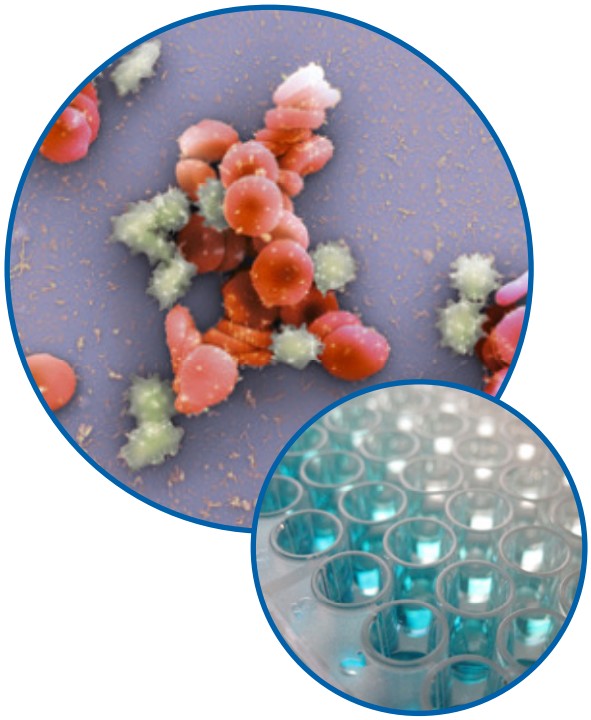


AdipoGen™

MANUAL

BAFF, Soluble (human) ELISA Kit (hypersensitive)

[BlyS, Soluble (human) ELISA Kit (hypersensitive)]

For research use only. Not for diagnostic use

Version 4 (March-15-2011)

Cat. No. AG-45B-0001-KI01

Table of Contents

1. Intended Use

The BAFF, Soluble (human) ELISA Kit (hypersensitive) is to be used for the *in vitro* quantitative determination of human BAFF (h) in serum, plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

BAFF (B cell activation factor of the TNF family, also known as BLyS or TALL1) is a key survival factor for peripheral B cells. BAFF is a homotrimeric type II transmembrane protein that can be proteolytically processed by furin to be released as soluble cytokine (1). Soluble BAFF adopts the classical trimeric form of the TNF-family ligand. However, BAFF has the unique property among the TNF-ligand to assemble as a 60-mer (2). BAFF is mainly produced by innate immune cells such as neutrophils, monocytes, macrophages, dendritic cells, follicular dendritic cells. T cells, activated B cells, some malignant B cells and also non-lymphoid cells like astrocytes, synoviocytes and epithelial cells can also produce BAFF. BAFF binds three distinct receptors (BAFF-R, TACI and BCMA) expressed predominantly on B cells, although activated T cells also express BAFF-R. BAFF is a master regulator of peripheral B cell survival, and together with IL-6, promotes Ig class-switching and plasma cell differentiation (1). Besides its major role in B cell biology, BAFF co-stimulates activated T cells. Deregulated expression of BAFF leads to autoimmune disorders in mice. In humans, elevated levels of soluble BAFF have been detected in the serum of patients with various autoimmune diseases (3), such as Sjögren's syndrome (4), Rheumatoid Arthritis (RA) (5), Multiple sclerosis (MS) (6) and Systemic Lupus Erythematosus (SLE) (7). BAFF is also increased levels in some lymphoid cancers (8).

3. General References

(1) Cracking the BAFF code: F. Mackay & P. Schneider; Nat. Rev. Immunol. 9, 491 (2009)

(2) TACI, unlike BAFF-R, is solely activated by oligomeric BAFF and APRIL to support survival of activated B cells and plasmablasts: C. Bossen, et al.; Blood 111, 1004 (2009)

(3) BAFF: a local and systemic target in autoimmune diseases: I. Moisini & A. Davidson; Clin. Exp. Immunol. 158, 155 (2009)

(4) B-cell tolerance breakdown in Sjögren's syndrome: focus on BAFF: M.M. Varin, et al.; Autoimmun. Rev. 9, 604 (2010)

(5) Concentrations of BAFF correlate with autoantibody levels, clinical disease activity, and response to treatment in early rheumatoid arthritis: S. Bosello, et al.; J. Rheumatol. 35, 1256 (2008)

(6) A BAFF antagonist suppresses experimental autoimmune encephalomyelitis by targeting cell-mediated and humoral immune responses: N.D. Huntington, et al. ; Int. Immunol. 18, 1473 (2006)

(7) B lymphocyte stimulator overexpression in patients with systemic lupus erythematosus: longitudinal observations: W. Stohl, et al.; Arthritis Rheum. 48, 3475 (2003)

(8) Serum BAFF predicts prognosis better than APRIL in diffuse large B-cell lymphoma patients treated with rituximab plus CHOP chemotherapy: S.J. Kim, et al.; Eur. J. Haematol. 81, 177 (2008)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human BAFF (BAFF) in serum, plasma and cell culture supernatant. A monoclonal antibody specific for BAFF has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, BAFF is recognized by the addition of a biotinylated monoclonal antibody specific for BAFF (DET). After removal of excess biotinylated antibody, streptavidine-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of BAFF in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial human BAFF Standard (lyophilized)	(5 µg)	(STD)
• 1 vial Detection Antibody	(30 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
• 2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with hBAFF Antibody	(6 x 16-well strips)	
• 2 plate Covers (plastic film)		
• 2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm, with the correction wavelength set at 540nm or 570nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.
- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 10 ml ELISA Buffer 10X + 90 ml water) to obtain ELISA Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:500 in ELISA Buffer 1X (4 µl AB + 2 ml ELISA Buffer 1X).

  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STRE-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human BAFF Standard (STD)** has to be reconstituted with 100 µl of distilled water.
  - This reconstitution produces a stock solution of 50 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C!*

  - Dilute the standard protein concentrate (STD) (**50 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.
  - Suggested standard points are:

    500 , 250 , 125 , 62.5 , 31.2 , 15.6 and 0 pg/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
500 ng/ml	10 µl of BAFF (STD)(50 µg/ml)	990 µl of ELISA Buffer 1X
10 ng/ml	10 µl of BAFF (STD)(0.5 µg/ml)	490 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
500 pg/ml	50 µl of BAFF (10 ng/ml)	950 µl of ELISA Buffer 1X
250 pg/ml	300 µl of BAFF (500 pg/ml)	300 µl of ELISA Buffer 1X
125 pg/ml	300 µl of BAFF (250 pg/ml)	300 µl of ELISA Buffer 1X
62.5 pg/ml	300 µl of BAFF (125 pg/ml)	300 µl of ELISA Buffer 1X
31.2 pg/ml	300 µl of BAFF (62.5 pg/ml)	300 µl of ELISA Buffer 1X
15.6 pg/ml	300 µl of BAFF (31.2pg/ml)	300 µl of ELISA Buffer 1X
0 pg/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample collection, storage and dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at - 20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma : Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at - 20°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma or **Cell Culture Supernatant** have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/30 to 1/40 dilutions of serum or plasma are recommended!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 16-well strips coated with BAFF antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted serum, plasma or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).**
☐	3. Cover the plate with plastic film and incubate for **3 hours at room temperature** (RT°C).
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the diluted Detection Antibody **(DET) (see 8.1 Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plastic film and incubate for **1 hour at RT°C**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plastic film and incubate for **30 min at RT°C**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB substrate solution **(TMB)**.
☐	12. Allow the color reaction to develop **at RT°C in the dark for 10-20 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 50 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
	! CAUTION: CORROSIVE SOLUTION !
☐	14. Measure the OD at 450 nm in an ELISA reader.

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding BAFF concentration (pg/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the BAFF concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of human BAFF in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

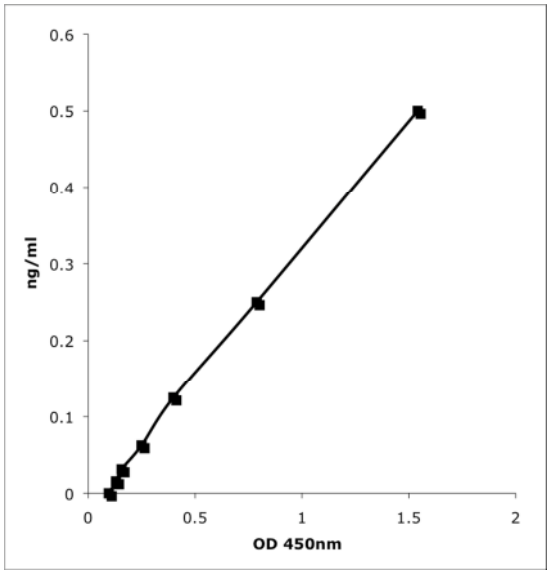


Figure: Standard curve

Standard hBAFF (pg/ml)	Optical Density (mean)
500	1.5415
250	0.788
125	0.3985
62.5	0.2495
31.2	0.156
15.6	0.13
0	0.096

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of BAFF that can be detected by this assay is 8 pg/ml. **NOTE:** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 15.6 pg/ml – 500 pg/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human BAFF**.** It does not cross-react with mouse BAFF.
Detection of BAFF (human) in biological fluids by this ELISA kit is abolished in the presence of a BAFF receptor such as TACI:Fc (AG-40B-0079)

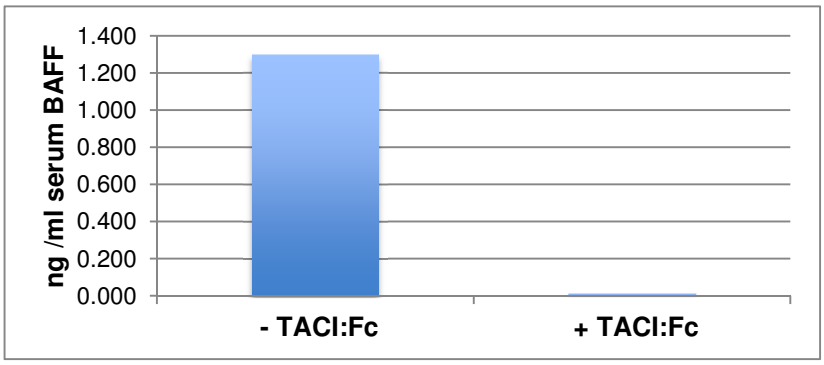


Figure: Specific quantitation of BAFF in human serum.

Method: Serum from a healthy patient was left untreated or treated with 1µg /ml of a BAFF receptor, TACI (h):Fc (h) (AG-40B-0079). BAFF levels were measured using the BAFF, Soluble (human) ELISA Kit (hypersensitive) (AG-45B-0001)

D. Intra-assay precision:

Five samples of known concentrations of human BAFF were assayed in replicates 16 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	1.71	0.14	8.18	16
A2	2.11	0.09	4.09	16
A3	2.48	0.10	4.09	16
A4	2.44	0.13	5.31	16
A5	1.71	0.13	7.33	16

E. Inter-assay precision:

Four samples of known concentrations of human BAFF were assayed in 4 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
B1	1.88	0.18	9.85	4
B2	1.82	0.18	9.93	4
B3	1.57	0.13	8.25	4
B4	1.76	0.23	12.9	4

F. Recovery:

When samples (serum or plasma) are spiked with known concentrations of human BAFF, the recovery averages 96% (range from 80% to 116%).

G. Linearity:

Different human serum and plasma (Li-Heparin) samples containing BAFF were diluted several fold (1/30 to 1/40) and the measured recoveries ranged from 92% to 107%.

H. Expected values:

BAFF levels range in plasma and serum from **0.5 to >5 ng /ml** (from healthy donors).

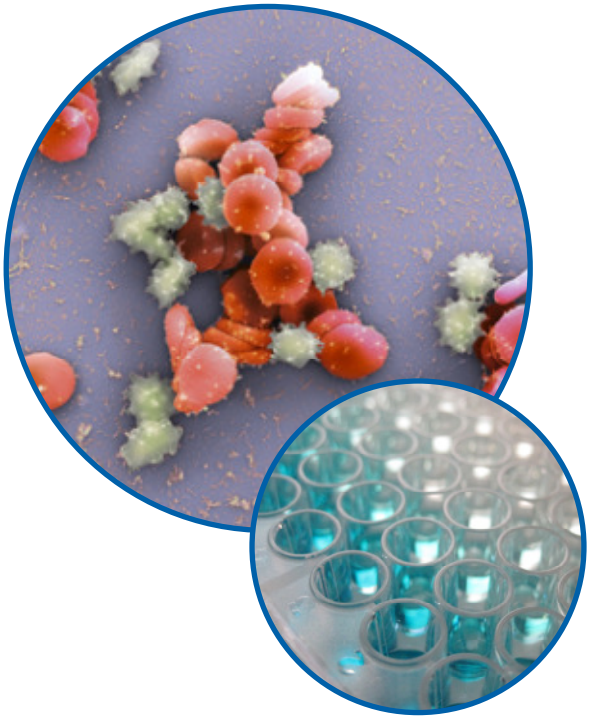
12. Technical Hints and Limitations

- It is recommended that all standards, controls and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37℃ before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of detector too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes



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