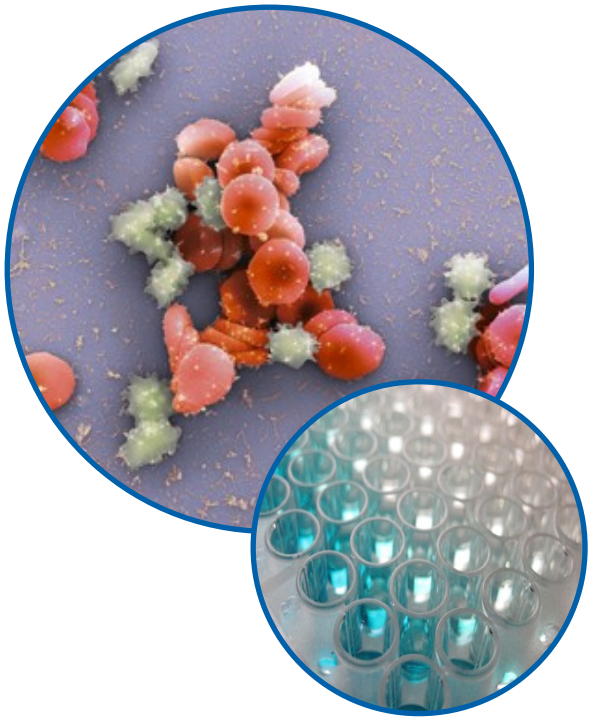


AdipoGen® LIFE SCIENCES

MANUAL

IL-36α (human) ELISA Kit

[IL-1F6 (human) ELISA Kit]

For research use only. Not for diagnostic use

Version 1 (June-07-2019)

Cat. No. AG-45B-0013-KI01

Table of Contents

1. Intended Use

The IL-36α (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human IL-36α in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. Introduction

IL-36α (IL-1F6), IL-36β (IL-1F8) and IL-36γ (IL-1F9) are members of the IL-1 cytokine family that bind to IL-36R (IL-1Rrp2) and IL-1RAcP, activating similar intracellular signals as IL-1 and are inhibited by IL-36Ra (1). IL-36α, β and γ cytokines, similarly to IL-1β, need to be processed to acquire their full agonist or antagonist activity (2). In their native form, IL-36 α, β and γ are 100–1000 times less active than their processed counterparts. Neutrophils proteases have been identified as the chief regulators of the processing of all the IL-36 family members, although with different specificity and affinity. IL-36α seems to be activated by both neutrophil Elastase and cathepsin-G, however, with differential patterns. The expression of IL-36 cytokines occurs mainly in the lung and skin and can be derived from diverse epithelial cell types including keratinocytes, bronchial epithelium as well as macrophages, monocytes and different T cell subsets.

IL-36 family members induce the production of pro-inflammatory cytokines, including IL-12, IL-1β, IL-6, TNF-α and IL-23, thus promoting neutrophil influx, dendritic cell (DC) activation, polarization of T helper type 1 (Th1) and IL-17-producing T cells (αβ T cells and γδ T cells) and keratinocyte proliferation (1). The IL-36 cytokines may represent potential targets for immune-mediated inflammatory conditions or, alternatively, could be used as adjuvants in vaccination.

IL-36α and IL-36β augmented IL-17A-mediated induction of antibacterial peptides in psoriasis (3). A significant increase in circulating and tissue levels of IL-36α occurs in Sjögren's syndrome patients (4). An increased expression of IL-36 α and IL-36 γ, but not IL-36 β has been observed in colonic inflammatory lesions from patients with inflammatory bowel disease, more prominently in ulcerative colitis (5, 6). An association between IL-36 and Th17 responses was also confirmed in Crohn's disease, although the expression of IL-36 in this population was significantly lower than in psoriasis (7). All members of the IL-36 subfamily are expressed during estrous cycle and pregnancy (8). IL-36α has also been involved in the prognosis of hepatocellular carcinoma, where its expression correlates negatively with tumor size, degree of differentiation, and level of vascular invasion. Correspondingly, high levels of IL-36α are positively correlated to the overall survival of patients (9).

3. General References

(1) IL-36 Cytokines: Regulators of inflammatory responses and their emerging role in immunology of reproduction: J.M. Murrieta-Coxca, et al.; Int. J. Mol. Sci. **20,** 1649 (2019)

(2) Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36α, β and γ) or antagonist (IL-36Ra) activity: J.E. Towne, et al.; J. Biol. Chem. **286**, 42594 (2011)

(3) Inter-Regulation of Th17 Cytokines and the IL-36 Cytokines In Vitro and In Vivo: Implications in Psoriasis Pathogenesis: Y. Carrier, et al.; J. Invest. Dermatol. **131**, 2428 (2011)

(4) Interleukin-36α axis is modulated in patients with primary Sjögren's syndrome: F. Ciccia, et al.; Clin. Exp. Immunol. **181**, 230 (2015)

(5) Increased expression of interleukin-36, a member of the interleukin-1 cytokine family, in inflammatory bowel disease: A. Nishida, et al.; Inflamm. Bowel Dis. **22**, 303 (2016)

(6) IL-36alpha expression is elevated in ulcerative colitis and promotes colonic inflammation: S.E. Russell, et al.; Mucosal Immunol. **9**,1193 (2016)

(7) Distinct expression of interleukin (IL)- 36alpha, beta and gamma, their antagonist IL-36Ra and IL-38 in psoriasis, rheumatoid arthritis and Crohn's disease: M.A. Boutet, et al.; Clin. Exp. Immunol. **184**, 159 (2016)

(8) Interleukin-1 family cytokines and their regulatory proteins in normal pregnancy and pre-eclampsia: J.H. Southcombe, et al.; Clin. Exp. Immunol. **181**, 480 (2015)

(9) Decreased expression of interleukin-36alpha correlates with poor prognosis in hepatocellular carcinoma: Q.Z. Pan, et al.; Cancer Immunol. Immunother. **62**, 1675 (2013)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human IL-36α in cell culture supernatants, serum and plasma. A monoclonal antibody specific for IL-36α 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, IL-36α is recognized by the addition of a biotinylated monoclonal antibody specific for IL-36α (DET). After removal of excess biotinylated antibody, streptavidin-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 IL-36α 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 IL-36α Standard (lyophilized)	(100 ng)	(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 IL-36α 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
- 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 1:500 in ELISA Buffer 1X (20 µl DET + 10 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 STREP-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 IL-36α Standard (STD)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - This reconstitution produces a stock solution of **1 µg/ml**. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

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

  - Dilute the standard protein concentrate (STD) (**1 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, 7.8 and 0 pg/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
10 ng/ml	10 µl of IL-36α (STD) (1 µg/ml)	990 µl of ELISA Buffer 1X
1 ng/ml	100 µl of IL-36α (STD) (10 ng/ml)	900 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
500 pg/ml	300 µl of IL-36α (STD) (1 ng/ml)	300 µl of ELISA Buffer 1X
250 pg/ml	300 µl of IL-36α (STD) (500 pg/ml)	300 µl of ELISA Buffer 1X
125 pg/ml	300 µl of IL-36α (250 pg/ml)	300 µl of ELISA Buffer 1X
62.5 pg/ml	300 µl of IL-36α (125 pg/ml)	300 µl of ELISA Buffer 1X
31.25 pg/ml	300 µl of IL-36α (62.5 pg/ml)	300 µl of ELISA Buffer 1X
15.6 pg/ml	300 µl of IL-36α (31.25 pg/ml)	300 µl of ELISA Buffer 1X
7.8 pg/ml	300 µl of IL-36α (15.6 pg/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, citrate or EDTA 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 ≤ -80°C for later use. Avoid repeated freeze/ thaw cycles.

Serum and 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, 1/20 to 1/60 dilutions of serum is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

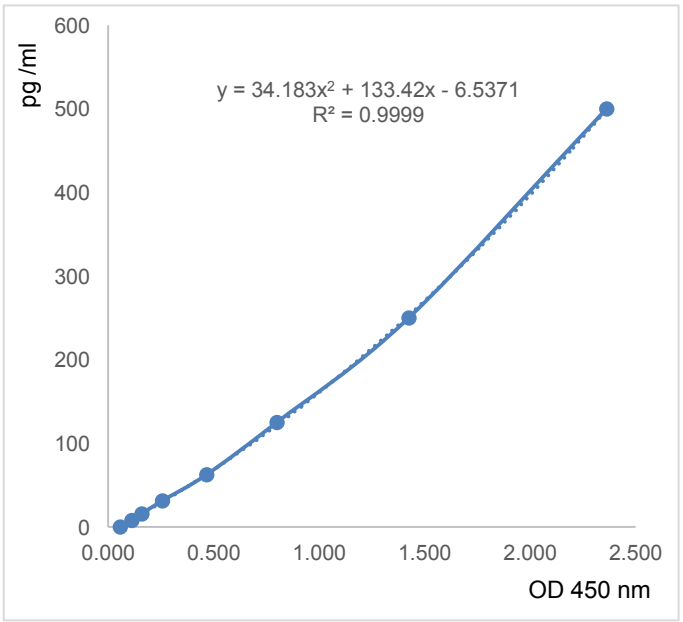
☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 16-well strips coated with* IL-36α *antibody when opened can be stored in the presence 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 plasma, serum 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 **2 h at RT.**
☐	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 h at RT**.
☐	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**.
☐	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 in the dark for 15-30 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 100 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 and sample and subtract the average blank value (obtained with the 0 pg/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding IL-36α concentration (pg/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the IL-36α concentrations of samples by interpolation of the regression curve formula 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 IL-36α in the sample.

10. Typical Data

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



Standard IL-36α (pg/ml)	Optical Density (mean)
500	2.362
250	1.425
125	0.799
62.5	0.466
31.75	0.256
15.9	0.159
7.8	0.111
0	0.056

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of IL-36α that can be detected by this assay is 4 pg/ml. **NOTE:** *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 7.8 pg/ml – 500 pg/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human IL-36α. It does not cross-react with human IL-36β and IL-36γ.

D. Intra-assay precision:

Four samples of known concentrations of human IL-36α were assayed in replicates 6 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	0.499	0.014	2.82	6
A2	0.122	0.006	4.60	6
A3	3.863	0.140	3.61	6
A4	0.254	0.008	3.17	6

E. Inter-assay precision:

Three samples of known concentrations of human IL-36α were assayed in 5 separate assays to test precision between assays.

Samples	Means (pg/ml)	SD	CV (%)	n
B1	0.254	0.004	1.48	5
B2	0.738	0.018	2.47	5
B3	1.250	0.050	3.96	5
B4	0.405	0.036	8.77	5

F. Recovery:

When samples (serum and plasma) are spiked with known concentrations of human IL-36α, the recovery averages 93% (range from 82% to 103%).

G. Linearity:

Different samples containing human IL-36α were diluted several fold (1/20 to 1/40 for sera and plasma) and the measured values average 94% (range from 82% to 112%).

G. Expected values:

Human IL-36α levels range in serum and plasma from <10pg/ml to >3ng /ml.

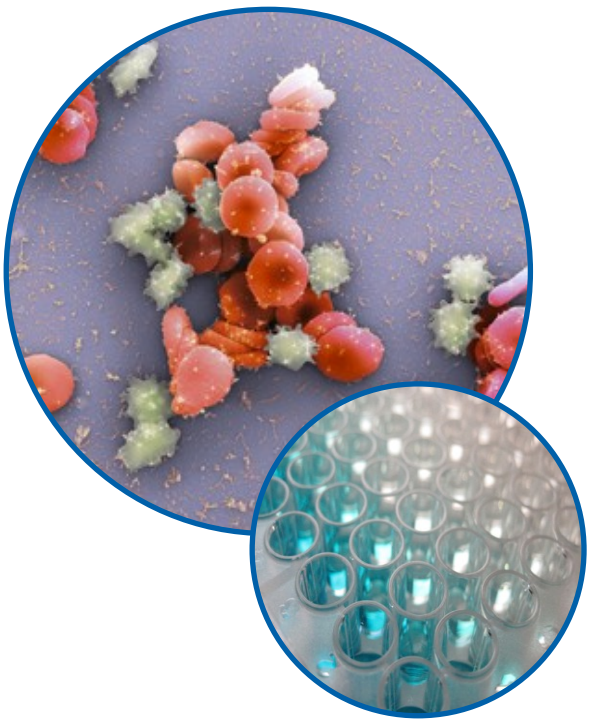
12. Technical Hints and Limitations

- It is recommended that all standards 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°C 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 STREP-HRP 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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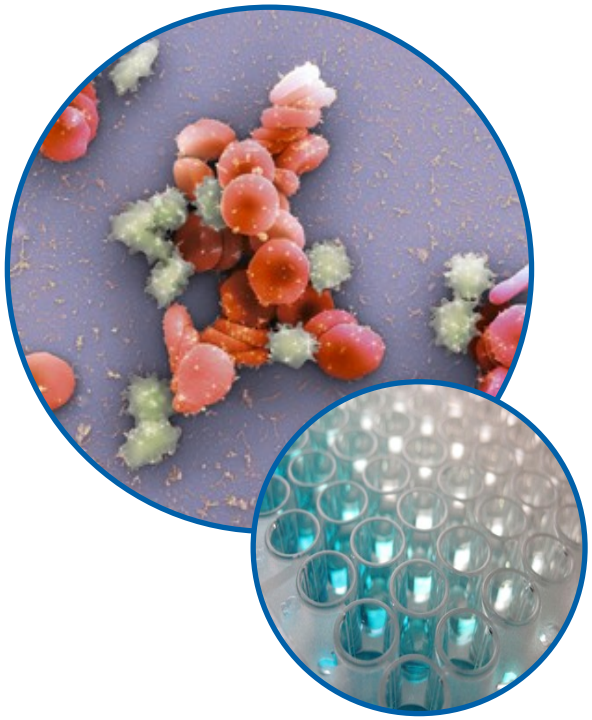

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MANUAL

IL-36γ (human) ELISA Kit

[IL-1F9 (human) ELISA Kit]

For research use only. Not for diagnostic use

Version 1 (September-27-2017)

Cat. No. AG-45B-0008-KI01

Table of Contents

1. Intended Use

The IL-36γ (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human IL-36γ in cell culture supernatants and serum. This ELISA Kit is for research use only.

2. Introduction

IL-36α (IL-1F6), IL-36β (IL-1F8) and IL-36γ (IL-1F9) are members of the IL-1 cytokine family that bind to IL-36R (IL-1Rrp2) and IL-1RAcP, activating similar intracellular signals as IL-1 and are inhibited by IL-36Ra (1). The expression of IL-36 cytokines occurs mainly in the lung and skin and can be derived from diverse epithelial cell types including keratinocytes, bronchial epithelium as well as macrophages, monocytes and different T cell subsets. IL-36γ expression is significantly induced upon TLR stimulation and induced in bronchial epithelium exposed to inflammatory stimuli suggesting that these proteins are involved in first-line defences against microorganisms (1,2). IL-36 family members induce the production of pro-inflammatory cytokines, including IL-12, IL-1β, IL-6, TNF-α and IL-23, thus promoting neutrophil influx, dendritic cell (DC) activation, polarization of T helper type 1 (Th1) and IL-17-producing T cells (αβ T cells and γδ T cells) and keratinocyte proliferation (1). The IL-36 cytokines may represent potential targets for immune-mediated inflammatory conditions or, alternatively, could be used as adjuvants in vaccination. Induction of IL-36γ in macrophages upon *M. tuberculosis* infection and its role in the release of antimicrobial peptides has been proposed (2). IL-36γ is also induced in the lung in various models of asthma and can be produced by bronchial epithelial cells in response to viral infection, smoke or inflammatory cytokines and plays an important role in asthmatic pulmonary inflammation (3). IL-36γ might be a potential biomarker of Inflammatory Bowel Disease (4,5), and is highly expressed in inflamed skin from psoriasis patients and in Allergic Contact Dermatitis (6). IL-36γ serum levels are enhanced and correlate to psoriasis severity and response to treatment with anti-TNF (7).

3. General References

(1) The emergence of the IL-36 cytokine family as novel targets for inflammatory diseases: P.T. Walsh & P.G. Fallon; Ann. N. Y. Acad. Sci. (Epub ahead of print) (2016)

(2) Role of Interleukin 36γ in Host Defense Against Tuberculosis: F. Ahsan, et al.; J. Infect. Dis. **214**, 464 (2016)

(3) Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma: Y.A. Bochkov, et al.; Mucosal Immunol. **3,** 69 (2010)

(4) IL-36 Receptor Promotes Resolution of Intestinal Damage: O. Medina-Contreras, et al.; J. Immunol. **196**, 34 (2016)

(5) Increased Expression of Interleukin-36, a Member of the Interleukin-1 Cytokine Family, in Inflammatory Bowel Disease: A. Nishida, et al.; Inflamm. Bowel Dis. **22**, 303 (2016)

(6) IL-36γ Is Involved in Psoriasis and Allergic Contact Dermatitis: A. Balato, et al.; J. Invest. Dermatol. **136**, 1520 (2016)

(7) IL-36γ (IL-1F9) is a biomarker for psoriasis skin lesions: A.M. D'Erme, et al.; J. Invest. Dermatol. **135**, 1025 (2015)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human IL-36γ in cell culture supernatants and serum. A monoclonal antibody specific for IL-36γ 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, IL-36γ is recognized by the addition of a biotinylated monoclonal antibody specific for IL-36γ (DET). After removal of excess biotinylated antibody, streptavidin-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 IL-36γ 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 IL-36γ Standard (lyophilized)	(1 µg)	(STD)
• 1 vial Detection Antibody	(20 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 IL-36γ 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
- 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 1:1000 in ELISA Buffer 1X (4 µl DET + 4 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 STREP-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 IL-36γ Standard (STD)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - This reconstitution produces a stock solution of 10 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

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

  - Dilute the standard protein concentrate (STD) (**10 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:

    250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 0 pg/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
100 ng/ml	10µl of IL-36γ (STD) (10 µg/ml)	990 µl of ELISA Buffer 1X
1 ng/ml	10µl of IL-36γ (STD) (100 ng/ml)	990 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
250 pg/ml	250µl of IL-36γ (STD) (1 ng/ml)	750 µl of ELISA Buffer 1X
125 pg/ml	300 µl of IL-36γ (250 pg/ml)	300 µl of ELISA Buffer 1X
62.5 pg/ml	300 µl of IL-36γ (125 pg/ml)	300 µl of ELISA Buffer 1X
31.25 pg/ml	300 µl of IL-36γ (62.5 pg/ml)	300 µl of ELISA Buffer 1X
15.6 pg/ml	300 µl of IL-36γ (31.25 pg/ml)	300 µl of ELISA Buffer 1X
7.8 pg/ml	300 µl of IL-36γ (15.6 pg/ml)	300 µl of ELISA Buffer 1X
3.9 pg/ml	300 µl of IL-36γ (7.8 pg/ml)	300 µl of ELISA Buffer 1X
0 ng/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.

Serum and 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, 1/2 dilution of serum is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

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* IL-36γ *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 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 **2 h at 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 h 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**.
☐	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 in the dark for 15-30 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 pg/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding IL-36γ concentration (pg/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the IL-36γ 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 IL-36γ in the sample.

10. Typical Data

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

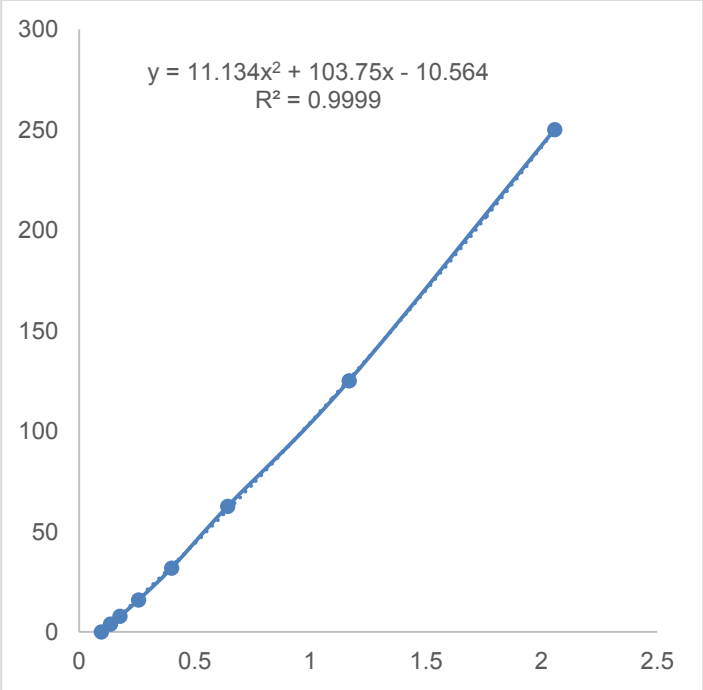


Figure: Standard curve

Standard IL-36γ (pg/ml)	Optical Density (mean)
250	2.0565
125	1.1675
62.5	0.642
31.75	0.3995
15.9	0.2565
7.8	0.176
3.9	0.135
0	0.0955

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of IL-36γ that can be detected by this assay is 3 pg/ml. **NOTE:** *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 3.9 pg/ml – 250 pg/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human IL-36γ. It does not cross-react with human IL-36α and IL-36β.

D. Intra-assay precision:

Four samples of known concentrations of human IL-36γ were assayed in replicates 8 times to test precision within an assay.

Samples	Means (pg/ml)	SD	CV (%)	n
A1	135.42	6.83	5.05	8
A2	64.28	2.50	3.89	8
A3	16.12	0.72	4.48	8
A4	285.22	11.75	4.12	8

E. Inter-assay precision:

Three samples of known concentrations of human IL-36γ were assayed in 4 separate assays to test precision between assays.

Samples	Means (pg/ml)	SD	CV (%)	n
B1	127.23	1.87	1.47	4
B2	29.86	1.01	3.40	4
B3	102.34	8.80	8.60	4
B4	83.78	7.74	9.25	4

F. Linearity:

Different samples containing human IL-36γ were diluted several fold (1/2 to 1/8) and the measured recoveries ranged from 95% to 106%.

G. Expected values:

Human IL-36γ levels range in serum from **< 3pg/ml (not detectable) (healthy patient) to > 100pg/ml (psoriasis patient)**.

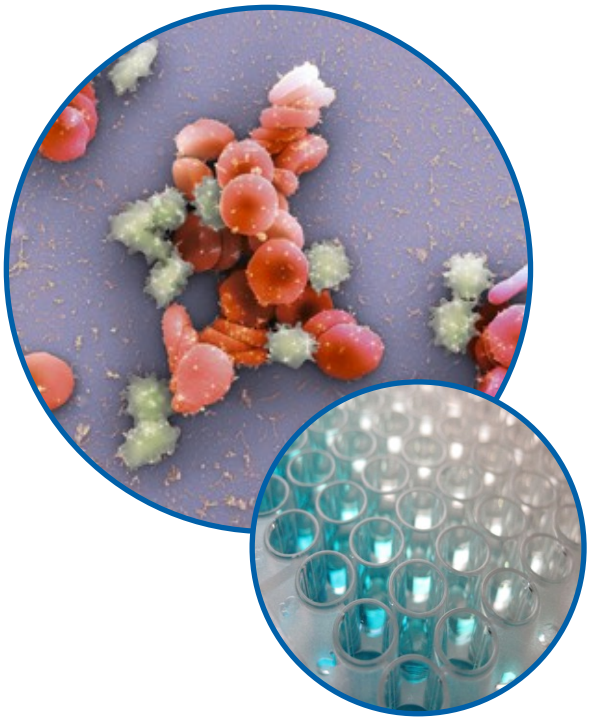
12. Technical Hints and Limitations

- 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°C 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 STREP-HRP 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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