Human IL-34 ELISA Kit

Instructions for use

Catalogue numbers:

1x48 tests: EA101863
1x96 tests: EA101864
2x96 tests: EA101865

For research use only

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Human IL-34 ELISA KIT

1. Intended use

The OriGenehuman IL-34 ELISA is a solid phase sandwich ELISA for the*in-vitro*qualitative and quantitative determination of IL-34 in cell culture supernatants, buffered solutions or human serum, plasma, or other body fluids. This assay will recognize both natural and recombinant human IL-34

This kit has been configured for research use only.

2. Introduction

2.1. Summary

IL-34 has been first described in 2008 as a cytokine that plays a role in macrophage differentiation and proliferation.
The IL-34 protein is composed of 241 amino acids, with a molecular mass of 39 kDa, secreted as homodimers. Human IL-34 is 71% identical to mouse IL-34 on the amino acid level.
This cytokine shares numerous common features with macrophage colony-stimulating factor (M-CSF), especially its receptor, partly explaining their functional overlap. It is expressed in various tissues and it is most abundant in the spleen.
Similar to M-CSF, IL-34 plays a major role in osteoclast genesis: in the presence of RANKL, IL-34 induces the proliferation and adhesion of osteoclast progenitors in vitro.
IL-34 has been implicated in some disease processes. For example,the role for IL-34 in rheumatoid arthritis (RA) has been evaluated. Joint fluid levels of IL-34 are increased in RA patients compared to controls with osteoarthritis. IL-34 expression levels in synovial membrane and joint fluid correlate with inflammation severity. In vitro, in the presence of TNF (and to a lesser degree of IL-1β or IL-17), rheumatoid synoviocytes produce IL-34 (whereas synoviocytes from osteoarthritic joints do not). Serum IL-34 levels are elevated in patients with RA.

2.2. Principle of the method

A capture Antibody highly specific for IL-34 has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of IL-34 in samples and known standards to the capture antibodies is completed and then any excess unbound analyte is removed. During the next incubation period the binding of the biotinylated anti-IL-34 secondary antibody to the analyte occurs. Any excess unbound secondary antibody is then removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of IL-34 present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of IL-34 in any sample tested.

3. Reagents provided and reconstitution

Reagents (Store@2-8^{o}C)	**Quantity 1x48 well kit** Cat no. EA101863	**Quantity 1x96 well kit** Cat no. EA101864	**Quantity 2x96 well kit** Cat no. EA101865	**Reconstitution**
96 well microtiter strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
IL-34 Standard: 5000 pg/ml	1 vial	2 vials	4 vials	Reconstitute as directed on the vial (see Assay preparation, section 8)
Standard Diluent (Buffer)	1 vial (25ml)	1 vial(25ml)	1vial (25ml)	10x Concentrate, dilute in distilled water (see reagent preparation, section 8)
Biotinylated Anti IL-34 Antibody	1 vial (0.4ml)	1 vial (0.4ml)	2 vials (0.4ml)	Dilute in Biotinylated Antibody Diluent (see Assay preparation, section 8)
Biotinylated Antibody Diluent	1 vial (7ml)	1 vial (7ml)	1 vial (13ml)	Ready to use
Streptavidin-HRP	1 vial (5µl)	2 vials (5µl)	4 vials (5µl)	Add 0.5ml of HRP diluent prior to use (see Assay preparation, section 8)
HRP Diluent	1 vial (23ml)	1 vial (23ml)	1 vial (23ml)	Ready to use
Wash Buffer	1 vial (10ml)	1 vial (10ml)	2 vials (10ml)	200x Concentrate dilute in distilled water (see Assay preparation, section 8)
TMB Substrate	1 vial (11ml)	1 vial (11ml)	1 vial (24ml)	Ready to use
H_2SO_4 stop reagent	1 vial (11ml)	1 vial (11ml)	2 vials (11ml)	Ready to use

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

Wash Buffer: Once prepared store at 2-8° C for up to 1 week
Standards : Once prepared use immediately and do not store
Biotinylated Secondary Antibody: Once prepared use immediately and do not store
Streptavidin-HRP: Once prepared use immediately and do not store

6. Specimen collection, processing & storage

Cell culture supernatants, serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Serum: Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1000 x g for 10 min and remove serum.

Plasma: EDTA, citrate and heparin plasma can be assayed. Spin samples at 1000 x g for 30 min to remove

Storage: If not analysed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- Laboratory gloves should be worn at all times
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance’s within 1 hour after completion of the assay
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- Follow incubation times described in the assay procedure
- Dispense the TMB solution within 15 min of the washing of the microtiter plate

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient microwell strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8^{o}C storage.

Example plate layout(example shown for a 6 point standard curve)

	Standards		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	5000	5000										
B	2500	2500										
C	1250	1250										
D	625	625										
E	312.5	312.5										
F	156.25	156.25										
G	Zero	Zero										
H												

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution.
Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder.Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2˚-8˚C for up to 1 week.

8.3. Preparation of Standard

Standard vials must be reconstituted with the volume of Standard Diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 5000pg/ml of IL-34. **Mix the reconstituted standard gently by inversion only**. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 5000 to 156.25 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 5000 pg/ml.
- Add 100µl of Standard Diluent to the remaining standard wells B1 and B2 to F1 and F2.
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 5000 pg/ml to 156.25 pg/ml.
- Discard 100µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.4. Preparation of Samples

Before testing, human serum or plasma samples have to be diluted 1:2 in Standard Buffer Diluent.

8.5. Preparation of Biotinylated Anti IL-34

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IL-34 with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of wells required	Biotinylated Antibody (µl)	Biotinylated Antibody Diluent (µl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360

8.6. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5µl vial with 0.5ml of HRP diluent **immediately before use.** Do-not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of wells required	Streptavidin-HRP (µl)	Streptavidin-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents as shown in section 8.
Note: Final preparation of Biotinylated anti-IL-34 (section 8.5) and Streptavidin-HRP (section 8.6) should occur immediately before use.

Assay Step		Details
1.	Addition	**Prepare Standard curve** as shown in section 8.3.
2.	Addition	Add 100µl of each **Standard, Diluted Sample and Zero** in duplicate to appropriate number of wells.
3.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours.**
4.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
5.	Addition	Add 50µl of diluted **Biotinylated anti-IL-34** to all wells.
6.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hours.**
7.	Wash	Repeat wash step 4.
8.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells.
9.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min.**
10.	Wash	Repeat wash step 4.
11.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells.
12.	Incubation	Incubate in the dark for **10-20 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil
13.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 13.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).		

***Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

10. Data Analysis

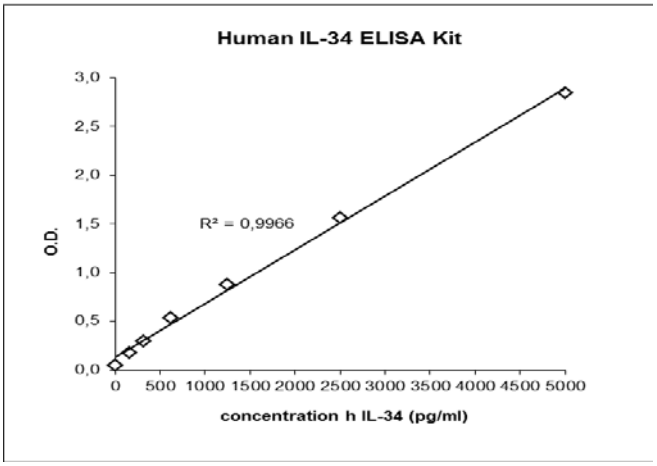
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding human IL-34 standard concentration on the horizontal axis.

The amount of IL-34 in each sample is determined by extrapolating OD values against IL-34 standard concentrations using the standard curve**.**

Example IL-34 Standard curve

Standard	IL-34 Conc (pg/ml)	OD (450nm) Mean	CV (%)
1	5000	2.846	5.7
2	2500	1.563	2.3
3	1250	0.880	1.9
4	625	0.539	2.6
5	312.5	0.299	7.8
6	156.25	0.179	4.0
Zero	0	0.049	5.8



Note: curve shown above should not be used to determine results. Every laboratory must run a standard curve for each set of microwell strips assayed.

For samples human serum or plasmas which have been diluted 1:2 according to the protocol, the calculated concentration should be multiplied by the dilution factor (x2).

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **afresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The minimum detectable dose of IL-34 was determined to be **39 pg/ml**. This was determined by adding 2 standard deviations to the mean OD obtained when the zero standard was assayed 36 times (in 6 different experiments).

12.2. Specificity

The assay recognizes natural and recombinant human IL-34. To define specificity of this ELISA, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (CD31, IL-10, IP-10, IL-31, IL-1α, IL-1β, GM-CSF, M-CSF, IL-29, MCP-1 and murine IL-34).

12.3. Precision

Intra-Assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates (3 duplicates) in 2 human pooled serum, 2 in culture media and 2 in standard diluent with samples containing different concentrations of IL-34.**The overall intra-assay coefficient of variation has been calculated to be 5.3%.**

Session	Sample	Mean IL-34 pg/ml	SD	CV%
Session 1	**Sample 1**	1871	141	**7.6**
	Sample 2	439	26	**6.0**
	Sample 3	1452	104	**7.2**
	Sample 4	170	11	**6.4**
	Sample 5	2959	160	**5.4**
	Sample 6	733	53	**7.2**
Session 2	**Sample 1**	1819	125	**6.9**
	Sample 2	469	34	**7.3**
	Sample 3	1446	22	**1.5**
	Sample 4	183	5	**2.8**
	Sample 5	2893	48	**1.7**
	Sample 6	796	37	**4.6**
Session 3	**Sample 1**	2148	37	**1.7**
	Sample 2	507	33	**6.4**
	Sample 3	1569	77	**4.9**
	Sample 4	212	15	**7.0**
	Sample 5	2745	103	**3.8**
	Sample 6	726	52	**7.1**

Inter-Assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments. Each assay was carried out with 6 replicates (3 duplicates) in 2 human pooled serum, 2 in culture media and 2 in standard diluent with samples containing different concentrations of IL-34.**The calculated overall coefficient of variation was 7.8%.**

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Mean IL-34pg/ml	1945	472	1489	188	2866	752
SD	181	40	89	21	137	53
CV%	**9.3**	**8.5**	**6.0**	**11.2**	**4.8**	**7.1**

12.4. Dilution Parallelism

Two spiked human serum and one spiked culture media with different levels of IL-34 were analysed at three serial two fold dilutions (1:2-1:8) with two replicates each. Recoveries ranged from 78% to 126% with an overall **mean recovery of 103%.**

12.5. Spike Recovery

The spike recovery was evaluated by spiking two concentrations of IL-34 in human serum and culture media in three experiments. Recoveries ranged from 67% to 108% with an overall **mean recovery of 91%.**

12.6. Stability

Storage Stability
Aliquots of spiked serum or culture media samples were stored at –20°C, 2-8°C, room temperature (RT) and at 37°C and the IL-34 level determined after 24h. No significant loss was observed.

Freeze-thaw Stability
Aliquots of spiked serum or culture media were stored frozen at –20°C and thawed up to 5 times and IL-34 level was determined. No significant loss was observed.

12.7. ExpectedValues

A panel of 10 sera and 10 plasmas of apparently healthy blood donors was tested for IL-34.
They were no detectable IL-34 levels found.

13. Bibliography

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Interleukin 34 expression is associated with synovitis severity in rheumatoid arthritis patients, Chemel and al, Ann Rheum Dis 2012;**71**:150–154.

Interleukin newcomers creating new numbers in rheumatology: IL-34 to IL-38, Chavel and al, Joint Bone Spine (2013)

Interleukin-34 produced by human fibroblast-like synovial cells in rheumatoid arthritis supports osteoclastogenesis, Hwang et al. Arthritis Research & Therapy 2012, 14:R14

14. Assay Summary

Total procedure length : 3h45

Add 100µl standard and diluted sample
↓
Incubate 2 hours at room temperature
↓
Wash three times
↓
Add 50µl Biotinylated IL-34 antibody
↓
Incubate 1 hour at room temperature
↓
Wash three times
↓
Add 100µl of Streptavidin-HRP
↓
Incubate 30min at room temperature
↓
Wash three times
↓
Add 100 µl of ready-to-use TMB
Protect from light. Let the colour develop for 10-20 min.
↓
Add 100µl of H_2SO_4
↓
Read Absorbance at 450 nm

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