Murine IL-17A PharmaGenie ELISA Kit

SKU:MODC0086

Instructions for use

For research use only

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Murine IL-17A PharmaGenie ELISA Kit

1. Intended use

The ELISA Genie IL-17A PharmaGenie ELISA kit is a solid phase sandwich ELISA for the *in vitro* qualitative and quantitative determination of IL-17A in supernatants, buffered solutions or serum and plasma samples and other fluids. This assay will recognise both natural and recombinant murine IL-17A.

PharmaGenie ELISA Kits from ELISA Genie are a premium range of pre-coated ELISA kits especially designed for scientists working in pharmaceutical, biotech & CRO sectors. PharmaGenie ELISA kits are produced using high quality monoclonal antibody pairs & optimized reagents that have been manufactured according ISO 9001:2000 quality systems and are excellent assays to help discover our future.

This kit has been configured for research use only.

2. Introduction

2.1. Summary

Interleukin-17 (IL-17, or IL-17A) is the founding member of a group of cytokines called the IL-17 family. IL17A, was originally identified as a transcript from a rodent T-cell hybridoma by Rouvier et al. in 1993.
IL-17A is involved in inducing and mediating proinflammatory responses, commonly associated with allergic responses and induces the production of many other cytokines (such as IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, TNF-α), chemokines (including IL-8, GRO-α and MCP-1) and prostaglandins (e.g. PGE_2) from many cell types (fibroblasts, endothelial cells, epithelial cells, keratinocytes and macrophages). IL-17A function is also essential to a subset of CD4+ T-Cells called T helper 17 (T_h17) cells.

2.2. Principle of the method

A capture Antibody highly specific for IL-17A has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of IL-17A 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-17A 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-17A 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-17A in any sample tested.

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3. Reagents provided and reconstitution

Reagents (Store@2-8ºC)	**Quantity 1x48 well kit**	**Quantity 1x96 well kit**	**Quantity 2x96 well kit**	**Reconstitution**
96 well microtitre strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
Standard: 500pg/ml	1	2	4	Reconstitute as directed on the vial (see Assay preparation, section 8)
Standard Diluent (Buffer)	1 (25ml)	1 (25ml)	2 (25ml)	10x Concentrate, dilute in distilled water (see reagent preparation, section 8)
Biotinylated anti-IL-17A	1 (0.4ml)	1 (0.4ml)	2 (0.4ml)	Dilute in biotinylated antibody diluent (see Assay preparation, section 8)
Biotinylated Antibody diluent	1 (7ml)	1 (7ml)	1 (13ml)	Ready to use
Streptavidin-HRP	1 (5µl)	2 (5µl)	4 (5µl)	Add 0.5ml of HRP diluent prior to use (see Assay preparation, section 8)
HRP Diluent	1 (23ml)	1 (23ml)	1 (23ml)	Ready to use
Wash Buffer	1 (10ml)	1 (10ml)	2 (10ml)	200x Concentrate dilute in distilled water (see Assay preparation, section 8)
TMB Substrate	1 (11ml)	1 (11ml)	1 (24ml)	Ready to use
H_2SO_4 stop reagent	1 (11ml)	1 (11ml)	2 (11ml)	Ready to use

4. Materials required but not provided

- Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm 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
Standard Diluent 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

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6. Specimen collection, processing & storage

Cell culture supernatants, human 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 particulates. Harvest plasma.

Storage: If not analyzed 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 of properly

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- 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 microtitre 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 aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 28ºC 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	500	500										
B	250	250										
C	125	125										
D	62.5	62.5										
E	31.25	31.25										
F	15.62	15.62										
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 Diluent Buffer

Add the contents of the vial (10x concentrate) to 225ml of distilled water before use.
This solution can be stored at 2-8ºC for up to 1 week.

8.4. Preparation of Standard

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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 500pg/ml of IL-17A. **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 500 to 15.6 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to well's A1 and A2, which provides the highest concentration standard at 500pg/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 500pg/ml to 15.6pg/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.

8.5. Preparation of Samples

Before testing, serum or plasmas samples have to be diluted 1:2 in standard buffer diluent.

8.6. Preparation of Biotinylated anti-IL-17A

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-IL-17A 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

8.7. 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

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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-17A (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 8.4
2.	Addition	Add 100µl of each**, 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 **1 hour(s)**
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-17A** to all wells
6.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **1 hour(s)**
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 **5-15 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 620 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*

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10. Data Analysis

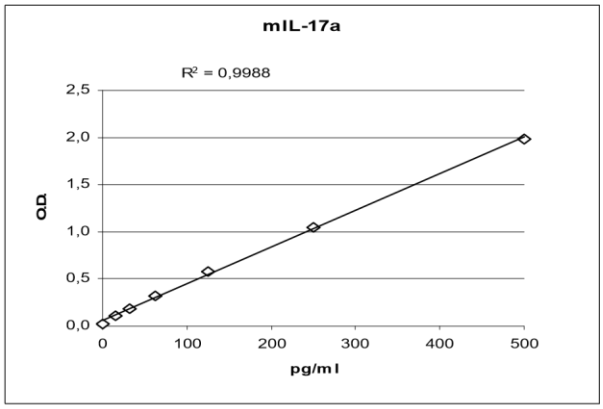
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding IL-17A standard concentration on the horizontal axis.

The amount of IL-17A in each sample is determined by extrapolating OD values against IL-17A standard concentrations using the standard curve**.**

Example mIL-17A Standard curve

Standard	IL-17A Conc	OD (450nm) mean	CV (%)
1	500	1.983	4.5
2	250	1.052	1.0
3	125	0.582	2.2
4	62.5	0.320	7.2
5	31.25	0.183	18.5
6	15.62	0.114	12.2
Zero	Zero	0.029	6.5



Note; curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.
For samples 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 **a fresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The sensitivity, minimum detectable dose of IL-17A using this ELISA Genie IL-17A ELISA kit was found to be **<4.6 pg/ml**. This was determined by adding 2 standard deviations to the mean OD obtained when the zero standard was assayed in 6 independent experiments.

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12.2. Specificity

The assay recognizes natural and recombinant murine IL-17A. To define specificity of this ELISA, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (human IL-17A, human IL-23, human IL-12p70 and p40, murine TNFα, murine IL-2, murine IFNγ, murine IL-10, murine IL-6, and murine GM-CSF)

12.3. Precision

Intra-assay

Reproducibility within the assay will be evaluated in three independent experiments. Each assay will be carried out with 6 replicates (3 duplicates) in 2 murine pooled serum, 2 in RPMI and 2 in standard diluent with samples containing different concentrations of IL-17A. 2 standard curves were run on each plate. **The overall intra-assay coefficient of variation has been calculated to be 1.9% .**

Session	***Sample***	**Mean** mIL-17A pg/ml	**SD**	**CV**
Session 1	***Sample 1***	395.75	5.96	**1.51**
	Sample 2	220.35	3.69	**1.68**
	Sample 3	283.35	2.98	**1.05**
	Sample 4	190.05	2.84	**1.49**
	Sample 5	364.26	6.84	**1.88**
	Sample 6	195.42	6.89	**3.53**
Session 2	***Sample 1***	394.56	6.48	**1.64**
	Sample 2	227.69	2.45	**1.08**
	Sample 3	262.71	3.66	**1.39**
	Sample 4	176.02	9.43	**5.36**
	Sample 5	351.86	0.46	**0.13**
	Sample 6	197.95	7.18	**3.63**
Session 3	***Sample 1***	390.29	7.51	**1.92**
	Sample 2	215.79	5.99	**2.78**
	Sample 3	294.34	1.18	**0.40**
	Sample 4	190.39	2.22	**1.17**
	Sample 5	346.03	9.99	**2.89**
	Sample 6	198.48	2.94	**1.48**

Inter-assay

Assay to assay reproducibility within one laboratory will be evaluated in three independent experiments by two technicians. Each assay will be carried out with 6 replicates (3 duplicates) in 2 murine pooled serum, 2 in RPMI and 2 in standard diluent with samples containing different concentrations of mIL-17A. 2 standard curves were run on each plate. **The calculated overall coefficient of variation was 0.2%.**

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	Sample 1	*Sample 2*	*Sample 3*	*Sample 4*	*Sample 5*	*Sample 6*
Mean IL-17A pg/ml	393.53	221.28	280.46	185.48	354.05	197.28
SD	0.4	0.35	0.11	0.31	1.15	0.32
CV	**0.1**	**0.16**	**0.04**	**0.17**	**0.32**	**0.16**

12.4. Dilution Parallelism

Two spiked murine serum with different levels of recombinant murine IL-17A were analysed at four serial two fold dilutions (1:2-1:16) with two replicates each. Recoveries ranged from 91% to 120% with an overall **mean recovery of 106%.**

12.5. Spike Recovery

The spike recovery was evaluated by spiking two concentrations of recombinant murine IL-17A in two murine serum and one culture media in three experiments. Recoveries ranged from 88% to 121% with an overall **mean recovery of 108%.**

12.6. Stability

Storage Stability
Aliquots of spiked serum sample and culture media were stored at –20°C, 2-8°C, room temperature (RT) and at 37°C and the murine IL-17A level determined after 24h. We observe 40% loss when stored at 2-8°C or 37°C and 67% after 24h at RT, when spiked in serum. As a result, we would advise that samples are stored at -20°C. When IL-17A is spiked in culture media, no significant loss is observed.

Freeze-thaw Stability

Aliquots of spiked serum were stored frozen at –20°C and thawed up to 5 times and murine IL-17A level was determined. We observed an average of 10% loss after one cycle, 30% loss after 3 cycles and 50% loss after 5 freeze-thaw cycles. When IL-17A is spiked in culture media, no significant loss is observed after 5 cycles of freezing and thawing.

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13. Assay Summary

Total procedure length: 2h45mn

Add sample, zero and diluted standard

↓

Incubate 1 hour at room temperature

↓

Wash three times

↓

Add 50µl of biotinylated detection 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 color develop for 5-15 mn.

↓

Add 100 H_2SO_4

↓

Read Absorbance at 450 nm

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