PRODUCT INFORMATION & MANUAL

Human IL-17A High Sensitivity ELISA

BMS2017HS

Enzyme-linked Immunosorbent Assay for quantitative detection of human IL-17A.

For research use only.
Not for diagnostic or therapeutic procedures.

eBioscience®
An Affymetrix Company

Human IL-17A High Sensitivity ELISA

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1 Intended Use

The human IL-17A ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human IL-17A. **The human IL-17A ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

2 Summary

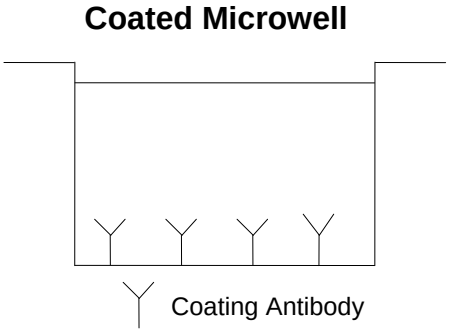
A new family of cytokines, Interleukin-17, has recently been defined that reveals a distinct ligand-receptor signaling system. There is high evidence for its importance in the regulation of immune responses. IL-17A was first characterised and six IL-17 family members (IL-17A-F) have subsequently been described. IL-17A, a homodimeric cytokine of about 32 kDa, is largely produced by activated memory T lymphocytes, but stimulates innate immunity and host defense. IL-17A and IL-17F both mobilize neutrophils partly through granulopoeisis and CXC chemokine induction, as well as increased survival locally. IL-17A and IL-17F production by T lymphocytes is regulated by IL-23 independent of T cell receptor activation.
The T help 1 (Th1) and Th2 cell classification has until recently provided the framework for understanding CD4(+) T cell biology and the interplay between innate and adaptive immunity. Recent studies have defined a previously unknown arm of the CD4(+) T cell effector response, the Th17 lineage. This subset of T cells produces interleukin 17, which is highly proinflammatory and induces severe autoimmunity. Whereas IL-23 serves to expand previously differentiated T(H)-17 cell populations, IL-6 and transforming growth factor-beta (TGF-beta) induce the differentiation of T(H)-17 cells from naive precursors.
Increasing evidence shows that IL-17 family members play an active role in inflammatory diseases, autoimmune diseases, and cancer. The IL-17 signaling system is operative in disparate tissues such as articular cartilage, bone, meniscus, brain, hematopoietic tissue, kidney, lung, skin and intestine. Thus, the evolving IL-17 family of ligands and receptors may play an important role in the homeostasis of tissues in health and disease beyond the immune system. Increased levels of IL-17 have been associated with several conditions, including airway inflammation, rheumatoid arthritis, intraperitoneal abscesses and adhesions, inflammatory bowel disease, allograft rejection, psoriasis, cancer and multiple sclerosis.

For literature update refer to **www.eBioscience.com**

3 Principles of the Test

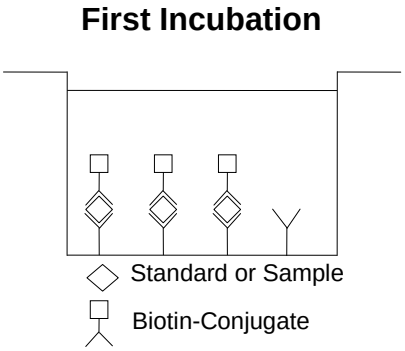
An anti-human IL-17A coating antibody is adsorbed onto microwells.

Figure 1



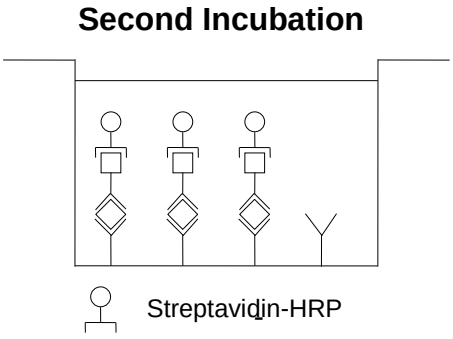
Human IL-17A present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human IL-17A antibody is added and binds to human IL-17A captured by the first antibody.

Figure 2



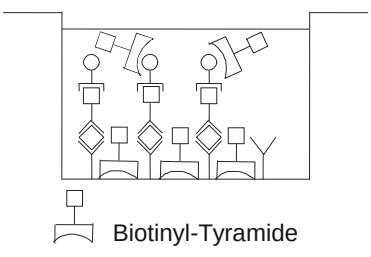
Following incubation unbound biotin-conjugated anti-human IL-17A antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human IL-17A antibody.

Figure 3



Following incubation unbound Streptavidin-HRP is removed during a wash step, and amplification reagent I (Biotinyl-Tyramide) is added to the wells.

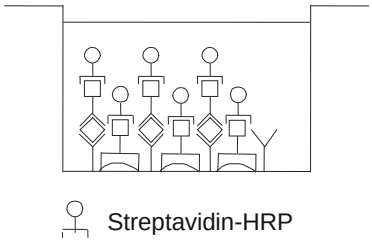
Figure 4



Following incubation unbound amplification reagent I is removed during a wash step and amplification reagent II (Streptavidin-HRP) is added.

Figure 5

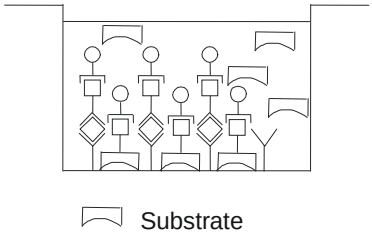
Fourth Incubation



Following incubation unbound amplification reagent II is removed during a wash step and substrate solution reactive with HRP is added.

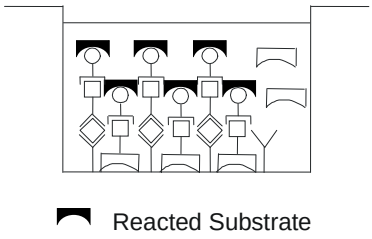
Figure 6

Fifth Incubation



A coloured product is formed in proportion to the amount of human IL-17A present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IL-17A standard dilutions and human IL-17A sample concentration determined.

Figure 7



4 Principle of Amplification Reaction

The amplification reaction is based upon PerkinElmer Life Sciences' TSA (Tyramide Signal Amplification) technology (see 15, References 1 and 2).
Amplification reagent I contains biotinyl-tyramide. HRP converts multiple biotinyl-tyramide molecules into highly reactive derivates (free radicals). These free radicals bind covalently to any protein in the well.
Thus, the amount of reacted biotinyl-tyramide is proportional to the amount of HRP in the well.
Following incubation unbound biotinyl-tyramide is removed during a wash step. Amplification reagent II contains Streptavidin-HRP, which binds to the biotin sites created during the biotinyl-tyramide reaction, thus multiplying the HRP molecules available at the surface for the substrate reaction.

5 Reagents Provided

1 aluminium pouch with a **Microwell Plate coated** with monoclonal antibody to human IL-17A

1 vial (70 µl) **Biotin-Conjugate** anti-human IL-17A monoclonal antibody

1 vial (150 µl) **Streptavidin-HRP**

2 vials human IL-17A **Standard** lyophilized, 200 pg/ml upon reconstitution

1 bottle (12 ml) **Sample Diluent**

1 vial (5 ml) **Assay Buffer Concentrate** 20x
(PBS with 1% Tween 20 and 10% BSA)

1 vial (7 ml) **Amplification Diluent Concentrate** (2x)

1 vial (75 µl) **Amplification Reagent I***

1 vial (90 µl) **Amplification Reagent II**

2 bottles (50 ml) **Wash Buffer Concentrate** 20x
(PBS with 1% Tween 20)

1 vial (15 ml) **Substrate Solution**

1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)

4 **Adhesive Films**

* reagent contains ethyl alcohol

6 Storage Instructions – ELISA Kit

Store kit reagents between 2° and 8°C.
Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels. 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, this reagent is not contaminated by the first handling.

7 Specimen Collection and Storage Instructions

Cell culture supernatant, serum and plasma (heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human IL-17A. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to 14.5).
Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

8 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

9 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.

- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

10 Preparation of Reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

10.1 Wash Buffer (1x)

Pour entire contents (50 ml) of the Wash Buffer Concentrate (20x) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

10.2 Assay Buffer (1x)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20x) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming.

Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.

Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

10.3 Biotin-Conjugate

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Biotin-Conjugate** solution with **Assay Buffer (1x)** in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	**Assay Buffer (1x)** (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

10.4 Streptavidin-HRP

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated **Streptavidin-HRP** solution with **Assay Buffer (1x)** in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	**Assay Buffer (1x)** (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

10.5 Human IL-17A Standard

Reconstitute **human IL-17A standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 200 pg/ml).

Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

The concentrated **human IL-17A standard** must be diluted 1:6.7 with Sample Diluent just prior to use in a clean plastic test tube according to the following dilution scheme:
100 µl reconstituted **human IL-17A standard** + 570 µl Sample Diluent. Shake gently to mix (concentration of standard = 30.00 pg/ml).

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate (see 11.d) or alternatively in tubes (see 10.5.1).

10.5.1 External Standard Dilution

Label 7 tubes, one for each standard point.

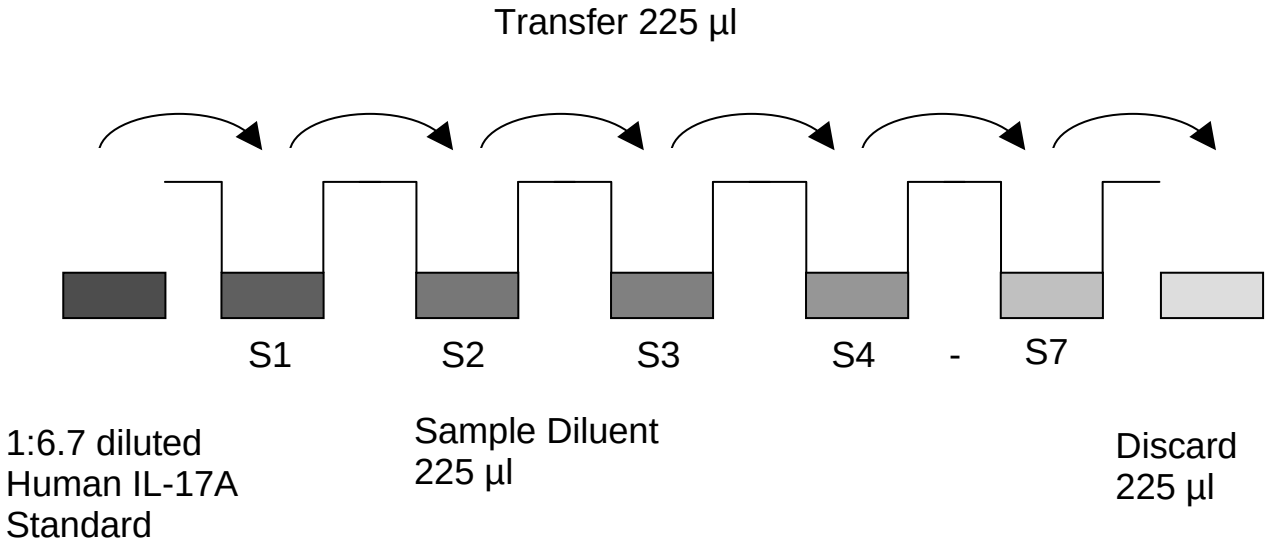
S1, S2, S3, S4, S5 S6, S7

Then prepare 2-fold serial dilutions for the standard curve as follows:
Pipette 225 µl of Sample Diluent into each tube.

Pipette 225 µl of diluted standard (concentration of standard = 30.00 pg/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 15.00 pg/ml).
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 8).

Sample Diluent serves as blank.

Figure 8



10.6 Amplification Diluent (1x)

Preparation of Amplification Diluent (1x) has to be done **immediately prior to use**. Make a 1:2 dilution of the concentrated **Amplification Diluent (2x)** as needed according to the following table:

Number of Strips	Amplification Diluent (2x) (ml)	Distilled Water (ml)
1 - 6	3	3
1 - 12	6	6

10.7 Amplification Solution I

Preparation of Amplification Solution I has to be done **immediately prior to application** on the plate.

Make a 1:600 dilution **of Amplification Reagent I** in **Amplification Diluent (1x)** as needed according to the following table:

Number of Strips	Amplification Reagent I (ml)	Amplification Diluent (1x) (ml)
1 - 6	0.01	5.99
1 - 12	0.02	11.98

Discard immediately any prediluted Amplification Solution I after usage.

10.8 Amplification Solution II

Preparation of Amplification Solution II has to be done **immediately prior to application** on the plate.
Centrifuge vial for a few seconds in a microcentrifuge before opening to collect liquid trapped in the lid.

Make a 1:200 dilution of **Amplification Reagent II** in Assay Buffer (1x) as needed according to the following scheme:

Number of Strips	Amplification Reagent II (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

Discard immediately any prediluted Amplification Solution II after usage.

11 Test Protocol

As this ELISA is a high sensitive system it is extremely important to stick exactly to the manual (washing procedure; chronology of / and preparation of solutions; incubation time) to obtain optimal test performance!

Please note: Amplification Solutions have to be prepared immediately prior to application on the plate! It is extremely important to wash the wells properly to obtain a good test performance!

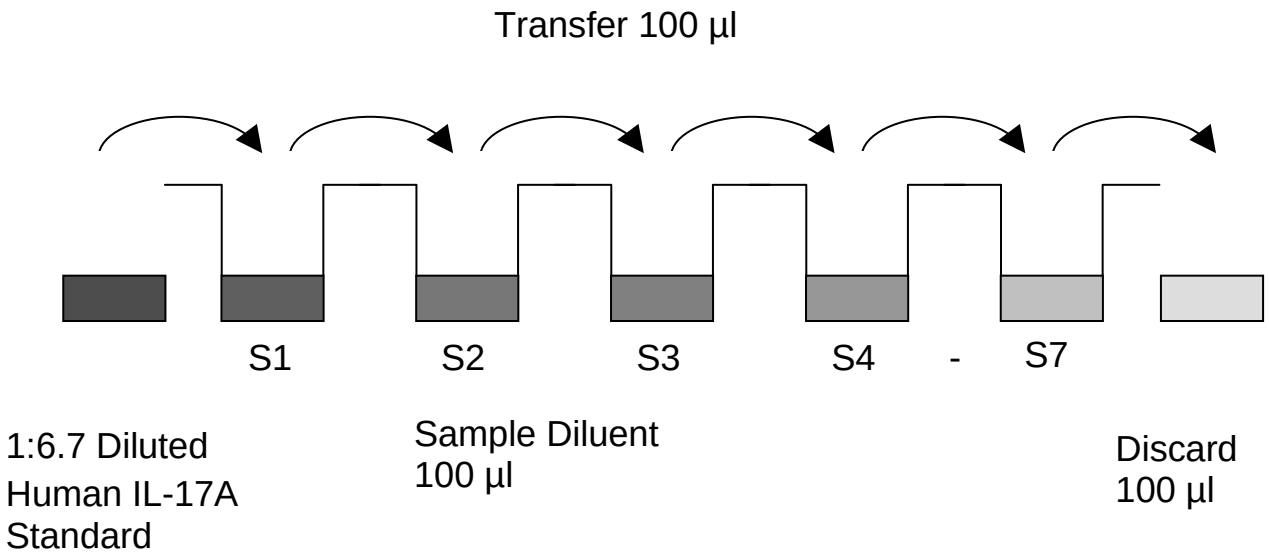
a. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

b. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate 10.3).

c. Wash the microwell strips twice with exactly 400 µl **Wash** Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Soaking is highly recommended between the washes to obtain a good test performance! Take care not to scratch the surface of the microwells.
After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. **Do not allow wells to dry**.

d. **<u>Standard dilution on the microwell plate</u>** (Alternatively the standard dilution can be prepared in tubes - see 10.5.1.):
Add 100 µl of Sample Diluent in duplicate to all **standard wells**.
Pipette 100 µl of prepared **standard** (see Preparation of Standard 10.5, concentration = 30 pg/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 15 pg/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 9). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human IL-17A standard dilutions ranging from 15.00 to 0.23 pg/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 9



In case of an **external standard dilution** (see 10.5.1.), pipette 100 µl of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1
Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	**2**	**3**	**4**
A	Standard 1 (15.00 pg/ml)	Standard 1 (15.00 pg/ml)	Sample 1	Sample 1
B	Standard 2 (7.50 pg/ml)	Standard 2 (7.50 pg/ml)	Sample 2	Sample 2
C	Standard 3 (3.75 pg/ml)	Standard 3 (3.75 pg/ml)	Sample 3	Sample 3
D	Standard 4 (1.88 pg/ml)	Standard 4 (1.88 pg/ml)	Sample 4	Sample 4
E	Standard 5 (0.94 pg/ml)	Standard 5 (0.94 pg/ml)	Sample 5	Sample 5
F	Standard 6 (0.47 pg/ml)	Standard 6 (0.47 pg/ml)	Sample 6	Sample 6
G	Standard 7 (0.23 pg/ml)	Standard 7 (0.23 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

e. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.

f. Add 50 µl of **Sample Diluent** to the **sample wells**.

g. Add 50 µl of each **sample** in duplicate to the **sample wells**.

h. Add 50 µl of **Biotin-Conjugate** to all wells.

i. Cover with an adhesive film and incubate at room temperature (18° to 25°C) over night, in the dark**.**

j. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP 10.4).

k. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

l. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.

m. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for exactly 1 hour on a microplate shaker in the dark. **(Shaking is absolutely necessary for an optimal test performance.)**

n. Prepare **Amplification Solution I** diluted in **Amplification Diluent (1x)** (see Preparation of Amplification Solution I 10.7) **immediately prior to use.**

o. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

p. Add 100 µl of **Amplification Solution I** to all wells, including the blank wells.

q. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for exactly 15 minutes on a microplate shaker in the dark. **(Shaking is absolutely necessary for an optimal test performance.)**

r. Prepare **Amplification Solution II** diluted in **Assay Buffer (1x)** (see Preparation of Amplification Solution II 10.8) **immediately prior to use.**

s. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

t. Add 100 µl of **Amplification Solution II** to all wells, including the blank wells.

u. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for exactly 30 minutes on a microplate shaker in the dark. **(Shaking is absolutely necessary for an optimal test performance.)**

v. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

w. Pipette 100 µl of **TMB Substrate Solution** to all wells.

x. Incubate the microwell strips at room temperature (18° to 25°C) for about 10-20 minutes in the dark. Avoid direct exposure to intense light.

   The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.

   It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

y. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

z. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

12 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human IL-17A concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human IL-17A for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human IL-17A concentration.
- **If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent). Thus concentrations read from the standard curve must be multiplied by the dilution factor (x 2 for samples).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human IL-17A levels. Such samples require further external predilution according to expected human IL-17A values with Sample Diluent in order to precisely quantitate the actual human IL-17A level.**
- It is suggested that each testing facility establishes a control sample of known human IL-17A concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 10. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 10

Representative standard curve for human IL-17A ELISA. Human IL-17A was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

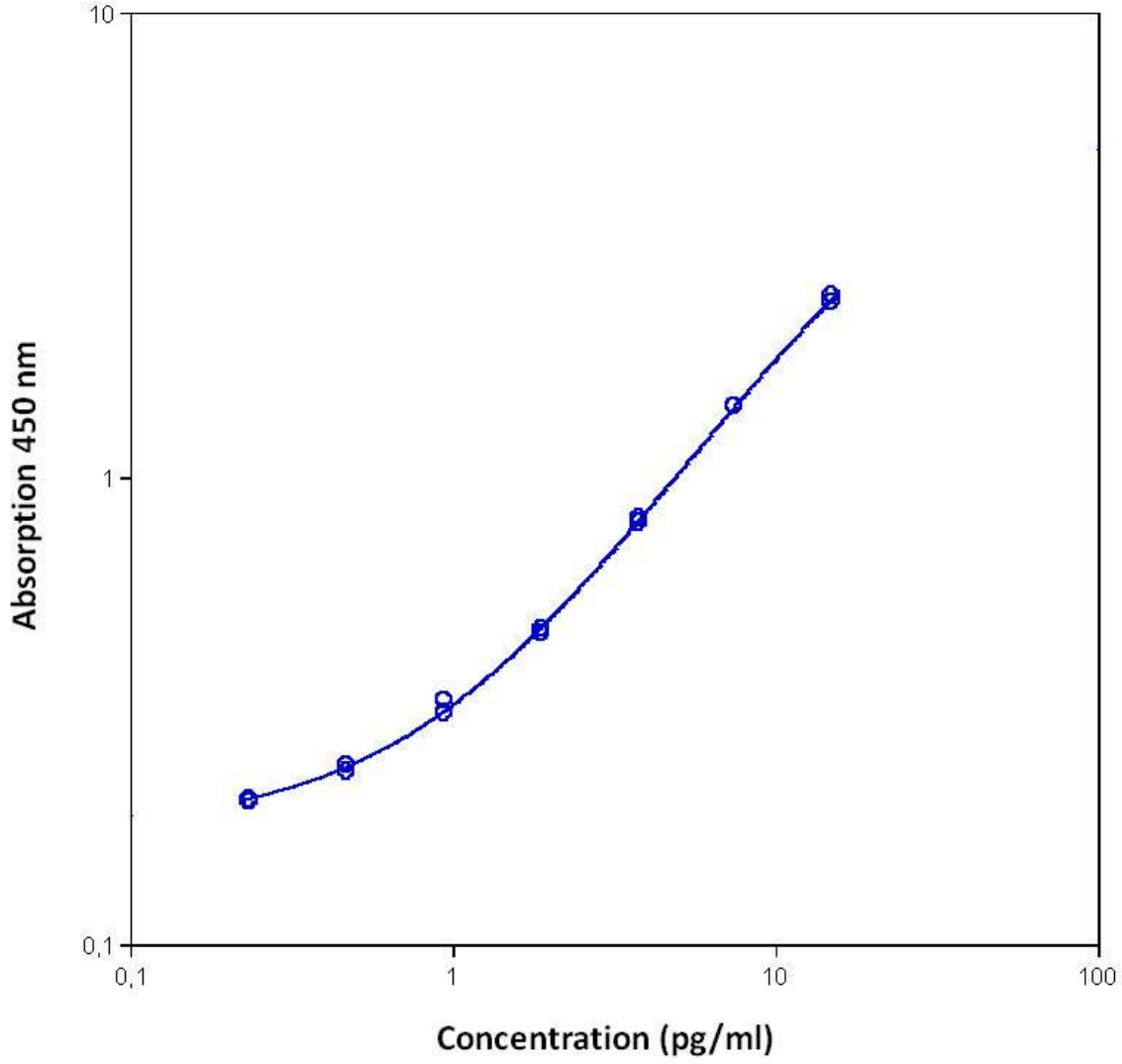


Table 2

Typical data using the human IL-17A ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	Human IL-17A Concentration (pg/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	15.00	2.457 2.380	2.418	1.6
2	7.50	1.425 1.435	1.430	0.3
3	3.75	0.803 0.823	0.813	1.2
4	1.88	0.465 0.474	0.469	1.0
5	0.94	0.336 0.314	0.325	3.5
6	0.47	0.243 0.236	0.239	1.6
7	0.23	0.205 0.203	0.204	0.3
Blank	0.00	0.146 0.146	0.146	0.2

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

13 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

14 Performance Characteristics

14.1 Sensitivity

The limit of detection of human IL-17A defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.01 pg/ml (mean of 4 independent assays).

14.2 Reproducibility

14.2.1 Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 4 serum samples containing different concentrations of human IL-17A. 2 standard curves were run on each plate. Data below show the mean human IL-17A concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 8.5%.

Table 3

The mean human IL-17A concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human IL-17A Concentration (pg/ml)	Coefficient of Variation (%)
1	1	20.6	2.2
	2	19.3	4.6
	3	18.6	10.1
2	1	18.4	4.2
	2	16.4	11.6
	3	16.2	8.3
3	1	6.1	5.4
	2	6.2	7.9
	3	5.8	5.1
4	1	3.9	4.1
	2	3.8	10.9
	3	3.8	8.2
5	1	2.1	14.4
	2	2.2	8.0
	3	1.9	10.2
6	1	1.3	13.5
	2	1.7	12.9
	3	1.7	9.3
7	1	4.2	8.8
	2	4.3	3.9
	3	3.8	8.9
8	1	2.1	10.9
	2	2.4	8.7
	3	2.1	11.4

14.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 4 serum samples containing different concentrations of human IL-17A. 2 standard curves were run on each plate. Data below show the mean human IL-17A concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6.3%.

Table 4
The mean human IL-17A concentration and the coefficient of variation of each sample

Sample	Mean Human IL-17A Concentration (pg/ml)	Coefficient of Variation (%)
1	19	5.0
2	17	7.3
3	6	3.5
4	4	2.2
5	2	6.6
6	4	6.8
7	2	8.8
8	2	10.4

14.3 Spike Recovery

The spike recovery was evaluated by spiking 3 levels of human IL-17A into serum, plasma (heparin) and cell culture supernatant. Recoveries were determined with 4 replicates each. The amount of endogenous human IL-17A in unspiked samples was subtracted from the spike values.

For the overall mean recovery see Table 5.

Table 5
The mean human IL-17A concentration and the coefficient of variation of each sample

Sample matrix	Spike high		Spike medium		Spike low	
	Mean (%)	Range (%)	Mean (%)	Range (%)	Mean (%)	Range (%)
Serum	84	(78-88)	84	(83-86)	79	(71-90)
Plasma (heparin)	74	(66-80)	86	(82-93)	91	(74-113)
Cell culture supernatant	90	(87-92)	70	(67-73)	59	(56-61)

14.4 Dilution Parallelism

Serum, plasma (heparin) and cell culture supernatant samples with different levels of human IL-17A were analysed at serial 2 fold dilutions with 4 replicates each.

For data see Table 6.

Table 6

Sample matrix	Recovery of Exp. Val.		
	Range (%)	Mean (%)	Range (%)
Serum	1:4 1:8 1:16	95 90 76	83 – 113 76 – 111 62 – 89
Plasma (heparin)	1:4 1:8 1:16	97 103 95	91 – 109 91 – 118 75 – 131
Cell culture supernatant	1:4 1:8 1 :16	97 85 81	84 – 114 74 – 110 79 – 83

14.5 Sample Stability

14.5.1 Freeze-Thaw Stability

Aliquots of spiked serum samples were stored at -20°C and thawed 5 times, and the human IL-17A levels determined. There was no significant loss of human IL-17A immunoreactivity detected by freezing and thawing.

14.5.2 Storage Stability

Aliquots of spiked serum samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human IL-17A level determined after 24 h.

There was no significant loss of human IL-17A immunoreactivity detected during storage under above conditions.

14.6 Specificity

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into serum. There was no crossreactivity detected with IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, TNF-α, TNF-β and IL-17.

14.7 Expected Values

There were no detectable human IL-17A levels found. Elevated human IL-17A levels depend on the type of immunological disorder.

15 References

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17 Reagent Preparation Summary

17.1 Wash Buffer (1x)

Add **Wash Buffer Concentrate** 20x (50 ml) to 950 ml distilled water.

Number of Strips	Wash Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	25	475
1 - 12	50	950

17.2 Assay Buffer (1x)

Add **Assay Buffer Concentrate** 20x (5 ml) to 95 ml distilled water.

Number of Strips	Assay Buffer Concentrate (20x) (ml)	Distilled Water (ml)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

17.3 Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in **Assay Buffer (1x):**

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

17.4 Streptavidin-HRP

Make a 1:200 dilution of **Streptavidin-HRP** in **Assay Buffer (1x):**

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

17.5 Human IL-17A Standard

Reconstitute lyophilized **human IL-17A standard** with distilled water. (Reconstitution volume is stated on the label of the standard vial.)
The concentrated **human IL-17A standard** must be diluted 1:6.7 with Sample Diluent.

17.6 Amplification Diluent (1x)

Prepare **Amplification Diluent (1x) immediately prior to use.**

Number of Strips	Amplification Diluent (2x) (ml)	Distilled Water (ml)
1 - 6	3	3
1 - 12	6	6

17.7 Amplification Solution I

Preparation of **Amplification Solution I** diluted 1:600 in **Amplification Diluent (1x)** has to be done **immediately prior to application** on the plate.

Number of Strips	Amplification Reagent I (ml)	Amplification Diluent (1x) (ml)
1 - 6	0.01	5.99
1 - 12	0.02	11.98

17.8 Amplification Solution II

Centrifuge vial for a few seconds in a micro-centrifuge before opening to collect liquid trapped in the lid. Preparation of **Amplification Solution II** diluted 1:200 in **Assay Buffer (1x)** has to be done **immediately prior to application** on the plate.

Number of Strips	Amplification Reagent II (ml)	**Assay Buffer (1x)** (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

18 Test Protocol Summary

Please note: Amplification Solutions have to be prepared immediately prior to application on the plate! It is extremely important to wash the wells properly to obtain a good test performance!

1. Determine the number of microwell strips required.
2. Prepare Biotin-Conjugate.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.
   Alternatively external standard dilution in tubes (see 10.5.1): Pipette 100 µl of these standard dilutions in the microwell strips.
5. Add 100 µl Sample Diluent in duplicate, to the blank wells.
6. Add 50 µl Sample Diluent to sample wells.
7. Add 50 µl sample in duplicate, to designated sample wells.
8. Add 50 µl Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate at room temperature (18° to 25°C) over night, in the dark.
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 6 times with Wash Buffer.
12. Add 100 µl diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate at room temperature (18° to 25°C) for exactly 1 hour on a microplate shaker in the dark. **(Shaking is absolutely necessary for an optimal test performance.)**
14. Prepare **Amplification Solution I** diluted in **Amplification Diluent (1x) immediately prior to application** on the plate.
15. Empty and wash microwell strips 6 times with Wash Buffer.
16. Add 100 µl Amplification Solution I to all wells.
17. Cover microwell strips and incubate at room temperature (18° to 25°C) for exactly 15 minutes on a microplate shaker in the dark. **(Shaking is absolutely necessary for an optimal test performance.)**
18. Prepare **Amplification Solution II** diluted in **Assay Buffer (1x) immediately prior to application** on the plate.
19. Empty and wash microwell strips 6 times with Wash Buffer

20. Add 100 µl Amplification Solution II to all wells.
21. Cover microwell strips and incubate at room temperature (18° to 25°C) for exactly 30 minutes on a microplate shaker in the dark. **(Shaking is absolutely necessary for an optimal test performance.)**
22. Empty and wash microwell strips 6 times with Wash Buffer.
23. Add 100 µl of TMB Substrate Solution to all wells.
24. Incubate the microwell strips for about 10-20 minutes at room temperature (18°to 25°C) in the dark.
25. Add 100 µl Stop Solution to all wells.
26. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed samples have been diluted 1:2 (50 µl sample + 50 µl Sample Diluent). Thus concentrations read from the standard curve must be multiplied by the dilution factor (x 2 for samples).