GE Healthcare

Amersham Tumour Necrosis Factor Alpha [(m)TNFa] Mouse, Biotrak ELISA System

96 Wells

Product Booklet

Code: RPN2718

Page Finder

1. Legal

Previously published 1995

http//www.gehealthcare.com/lifesciences

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2. Handling

2.1 Safety warnings and precautions

Warning: *For research use only.* Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

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.

2.2 Storage

Store at 2–8°C.

2.3 Expiry

The expiry date is stated on the package and will normally be at least 4 weeks from the date of despatch.

3. Components of the assay system

This pack contains the following assay components and provides sufficient material for 96 wells.

All reagents are stored refrigerated at 2–8°C. Refer to the expiry date on the kit box.

(m)TNFα microplate -
96 well polystyrene microplate (12 strips of 8 wells) coated with an antibody against mouse TNFα.

Biotinylated antibody reagent - antibody against mouse TNFα conjugated to biotin, with preservative, 8 ml.

Streptavidin-HRP concentrate - streptavidin conjugated to HRP, with preservative, 50 µl.

Streptavidin-HRP dilution buffer - dilution buffer, with preservative, 14 ml.

(m)TNFα standard - recombinant mouse TNFα, lyophilized, 2 vials.

Standard diluent - diluent, with preservative, 12 ml.

Wash buffer concentrate -
30-fold concentrated solution, with preservative, 50 ml.

Pre-mixed TMB substrate solution - substrate solution, 12-13 ml.

Stop solution - <1% sulfuric acid, 13-15 ml

Plate covers - adhesive strips, 4.

4. Additional materials and equipment required

The following materials and equipment are required but not supplied:

- Pipettes or pipetting equipment with disposable tips (50 µl, 100 µl and 1.00 ml)
- Disposable polypropylene test tubes - do not use polystyrene, polycarbonate or glass
- Measuring cylinder, 2 l and 20 ml
- Distilled or deionized water
- Plate reader capable of reading at 450 nm
- Reagent reservoirs for use with multi-channel pipettes

Optional equipment

Assays may be performed with commercially available microplate washers to aid convenience and assay throughput.

5. Description

The Biotrak™ mouse tumour necrosis factor a ELISA system from GE Healthcare provides a simple, specific, reliable and precise quantitative determination of (m)TNFα in cell culture supernatants and serum.

The assay system is based on a solid phase ELISA, which utilizes an antibody for (m)TNFα bound to the wells of a microplate (12 x 8 well strip format) together with a biotinylated antibody to (m)TNFα and streptavidin conjugated to horseradish peroxidase. Although the Biotrak (m)TNFα immunoassay contains recombinant (m)TNFα and antibodies raised against recombinant (m)TNFα it has been shown to quantitate accurately both natural (m)TNFα and recombinant (m)TNFα.

(m)TNFα can be measured in the range 50–2450 pg/ml (2.5–122.5 pg/well) in less than 4 hours using the protocol provided with the kit. Each pack contains sufficient material for 96 wells. If one standard curve is constructed, 44 unknowns can be measured in duplicate.

- High sensitivity – 10 pg/ml (0.5 pg/well)
- Same day protocol
- Pre-coated microplate
- Specific for (m)TNFα

Summary of the assay

This assay employs a quantitive *in vitro* enzyme linked immunosorbent technique. An antibody specific for (m)TNFα has been coated on to the microplate provided in the kit. Samples are pipetted into the wells along with biotinylated antibody reagent. If present, the (m)TNFα is bound by both the immobilized and the biotinylated antibody. After washing away any unbound sample proteins and biotinylated antibody, a streptavidin HRP conjugate is added to the

wells. Any (m)TNFα which was bound by both the immobilized and the biotinylated antibody during the first incubation will be bound by the streptavidin conjugate. Following a wash to remove any unbound conjugate, a substrate solution is added to the wells and color develops in proportion to the amount of (m)TNFα bound in the initial step.

In addition to the samples to be tested, a series of wells is prepared using known concentrations of the (m)TNFα standard. A curve, plotting the optical density versus the concentration of (m)TNFα in these standard wells, is prepared. By comparing the optical density of the samples to this standard curve, the concentration of the (m)TNFα in the unknown samples is then determined.

6. Critical parameters

- Allow samples and all reagents to reach room temperature prior to performing the assay. Do not use water baths to thaw samples or reagents.
- Mix samples and all reagents thoroughly before use.
- Avoid excessive foaming of reagents. Also avoid exposure of reagents to excessive heat or light during storage and incubation.
- Avoid handling the tops of the wells both before and after filling.
- Standards and samples should be assayed in duplicate.
- Run a separate standard curve for each assay.
- The total dispensing time for each plate should not exceed 20 minutes.
- Use only coated wells from the same reagent batch for each assay. Also do not mix reagents from different kit lots.
- For sample and conjugate incubations a humidified incubator may be used to help prevent evaporation loss due to incomplete plate sealing.

7. Sample preparation

Cell culture supernatants

Centrifuge to remove any particulate material and store at -15°C to -30°C.

Avoid freeze-thaw cycles.

Serum

Serum samples should be allowed to clot at room temperature. Immediately after clotting, spin down. Specimens should be clear and non-hemolyzed whenever possible. If samples contain particulate matter, clarify by centrifugation before testing.

Serum and culture supernatant samples that are to be assayed within 24 hours should be stored at 2–8°C. Specimens to be stored for longer periods of time should be frozen at -70°C to avoid loss of biologically active cytokine. Avoid freezing and thawing samples more than once.

Dilution of test samples

If it is suspected that the (m)TNFα gamma concentration of a sample exceeds the highest point of the standard curve, prepare one or more five-fold dilutions of the test sample. Mix thoroughly between dilutions and before assaying.

8. Assay procedure

8.1. Reagent preparation

Wash buffer concentrate

Any precipitate formed during storage will redissolve upon dilution. Dilute 30-fold with distilled or deionized water to prepare 1500 ml of wash buffer. Store at 2–8°C until the expiry date of the kit. Do not use wash buffer if it becomes visibly contaminated on storage.

Streptavidin-HRP solution

No more than 15 minutes prior to use, prepare the exact quantity of streptavidin-HRP solution required. Do not store prepared streptavidin-HRP solution. The streptavidin-HRP concentrate may require spinning down to force the entire contents to the bottom of the vial. Add 30 µl of streptavidin-HRP concentrate to 12 ml streptavidin-HRP dilution buffer in a 15 ml plastic tube and mix gently. The streptavidin-HRP solution is now ready for use.

If running partial plates, use only the amount of streptavidin-HRP solution required for the number of strips being run. Use 2.5 µl of streptavidin-HRP concentrate and 1 ml of streptavidin-HRP dilution buffer per strip being run.

(m)TNFα standard

It is important that the diluent selected for reconstitution and dilution of the standard reflects the environment of the samples being measured. Standard diluent will be suitable for the serial dilution of standards for serum determinations. If the samples are cell culture supernatants, the culture media will be suitable for preparation of the standard curve.

Reconstitute the (m)TNFa standard with distilled or deionized water for serum samples, and culture media for cell culture supernatants. Reconstitution volume is stated on the standard vial label. This

reconstitution produces a stock solution of 2450 pg/ml. Mix by gently inverting the vial. Use this stock solution to produce a dilution series, as described below, within the range of this assay (50–2450 pg/ml). Use standards within 15 minutes of dilution.

8.2. Preparation of standard curve

The reconstituted 2450 pg/ml (m)TNFα is the first point of the standard curve. For the remaining points, prepare seven-fold serial dilutions as follows: label two tubes, one tube for each of the additional dilutions: 350 pg/ml and 50 pg/ml. Pipette 600 µl of appropriate diluent into each tube. Pipette 100 µl of reconstituted (m) TNFα standard into the first tube labelled 350 pg/ml and mix. Pipette 100 µl of this dilution into the second tube labelled 50 pg/ml and mix. These concentrations, 2450 pg/ml, 350 pg/ml and 50 pg/ml provide the standard curve points.

8.3. Running partial plates

This ELISA provides the flexibility to run two partial plates on separate occasions. Decide the number of strips you wish to run, leaving the strips to be used in the frame. Remove the unnecessary strips and store them in the foil pouch with the desiccant provided at 2–8°C, making sure the foil pouch is sealed tightly.

When adding the TMB substrate solution, pour out from the bottle only the amount needed to run a partial plate. Do not combine left over substrate with that reserved for the remainder of the plate. Care must be taken to ensure that the remaining TMB substrate solution is not contaminated. If the substrate solution is bright blue prior to use, it has been contaminated. **DO NOT USE.**

8.4. Assay protocol

1. Prepare assay reagents and working standards as described in the previous sections.

2. Set up the microplate with sufficient wells to enable the running of all standards and samples as required (see figure 1).
3. Remove excess microplate strips from the frame and store in the resealable foil bag with the desiccant provided.
4. Add 50 µl of standard or sample per well, in duplicate.
5. Add 50 µl of biotinylated antibody reagent (has a cloudy white appearance). Cover with adhesive strip provided and incubate for 2 hours at room temperature.
6. Aspirate or decant each well and wash, repeating the process four times for a total of five washes. Wash vigorously by filling each well with wash buffer (400 µl) using a washbottle, pipette or manifold dispenser. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining wash buffer by inverting the plate and blotting it against clean paper towelling. Squeeze the sides of the plate when decanting to ensure that all strips remain securely in the frame.
7. Add 100 µl of streptavidin-HRP conjugate. Cover with a new adhesive strip and incubate for 30 minutes at room temperature.
8. Repeat the aspiration/wash step as in step 6.
9. Add 100 µl of TMB substrate solution into each well, incubate for 30 minutes at room temperature. If the substrate solution is bright blue prior to use, do not use. **THE PLATE SHOULD BE DEVELOPED IN THE DARK**. Do not cover the plate with aluminium foil.
10. Add 100 µl of stop solution to each well.
11. Determine the optical density of each well within 30 minutes, using a spectrophotometer set to 450 nm.

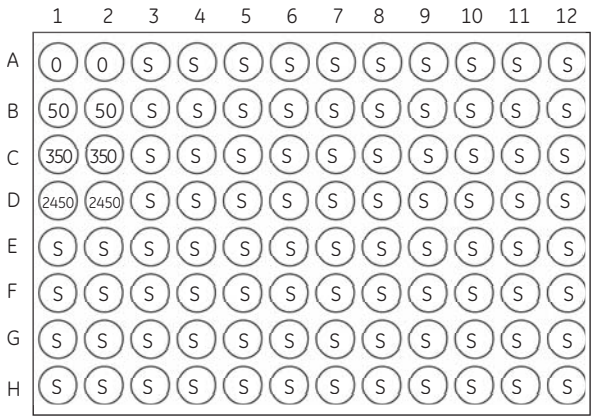


Figure 1. Recommended positioning of standard (0–2450 pg/ml) and sample wells (S).

Table 1. Assay protocol (all volumes are in microlitres)

	Zero standard (B_0)	Standards	Samples
Biotinylated antibody reagent	50	50	50
Standard	–	50	–
Standard diluent or cell culture media	50	–	–
Sample	–	–	50
Cover plate, incubate at room temperature for 2 hours. Aspirate/decant and vigorously wash all wells five times with 400 µl wash buffer.			
Wash the plate x 4 with wash buffer			
Streptavidin-HRP solution	100	100	100
Cover plate, incubate at room temperature for 30 minutes. Aspirate/decant and vigorously wash all wells five times with 400 µl wash buffer.			
Substrate	100	100	100
Incubate at room temperature for 30 minutes in the dark.			
Stop solution	100	100	100
Determine optical density at 450 nm within 30 minutes.			

9. Data processing

9.1. Calculation of results

Average the duplicate readings for each standard, control and sample and subtract the zero standard optical density.

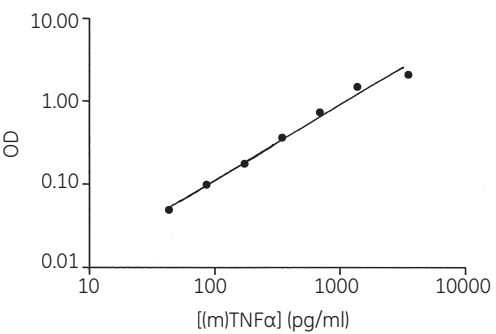
Plot these averaged absorbance values for each of the standard values versus the corresponding concentration of the standards. The data can be linearized by using a log/log plot and regression analysis may be applied to the log transformation.

Figure 2 shows such a plot of the data from table 2. The standard curve, with 7 points plotted, is provided for illustration purposes only. A standard curve should be generated for each set of samples to be assayed. The protocol describes the generation of a 4 point curve (zero included) by dilution of a stock standard. This allows for the measurements of 42 unknowns in duplicate.

If a test sample has been diluted prior to assay, account for the dilution factor in calculation of results.

9.2. Typical assay data

Figure 2. Standard curve



The following data (table 2) were obtained for a standard curve using the protocol provided, with an extended dilution series to demonstrate the linearity of the assay curve.

Table 2. Typical assay data

Tube	Optical density	Zero standard subtracted
Zero standard	0.1	–
43 pg/ml standard	0.1495	0.0495
86 pg/ml standard	0.199	0.099
172 pg/ml standard	0.277	0.177
344 pg/ml standard	0.466	0.366
687.5 pg/ml standard	0.841	0.741
1375 pg/ml standard	1.598	1.498
3500 pg/ml standard	2.203	2.103

10. Additional information

10.1. Specificity

This assay recognizes both natural and recombinant (m)TNFα. It does not cross react with (m)IL-2, (m)IL-3, (m)IL-4, (m)IL-5, (m)IL-6, (m)GM-CSF, (m) IFNγ or (h)TNFα.

10.2. Reproducibility

Within-assay precision

The within-assay coefficient of variation of the ELISA has been determined to be <10%.

Between-assay precision

The between-assay coefficient of variation of the ELISA has been determined to be <10%.

10.3. Sensitivity

The minimum detectable dose of biologically active (m)TNFα was determined to be 10 pg/ml (0.5 pg/well), by adding two standard deviations to the optical density value of zero and calculating the corresponding concentration from the standard curve.

10.4. Calibration

The standard in this ELISA is calibrated to the NIBSC reference 85/532

One (1) pg of Biotrak standard = 3 NIBSC pg

10.5. Recovery

Serum recovery in the ELISA has been determined by spiking recombinant cytokine into neat pooled mouse serum and comparing it with spiked standard diluent control. The values opposite are typical recoveries:

Control (pg/ml)	Recovery
135 pg/ml	97%
1182 pg/ml	81%

11. Background

Mouse tumor necrosis factor alpha ((m)TNFα) is a 17–18 kDa glycosylated protein produced by leukocytes and other cell types in response to a variety of infectious and activating agents. Mouse TNFα is approximately 80% homologous to human TNFα at the amino acid level. TNFα was identified by researchers investigating molecules mediating cachexia (wasting) and tumor cytotoxicity and is now known to play a central role in the regulation of inflammation, immunity and host defense. The rapid production of this potent cytokine in response to microbial infections can lead to both beneficial effects locally and lethal effects systemically, highlighting the dual nature of TNFα. TNFβ, or lymphotoxin, is a functionally-related cytokine that shares a common receptor system with TNFα.

TNFα signalling is mediated by two distinct TNF receptors, termed type I (55–60 kDa) and type II (75–80 kDa), both of which bind TNFα and TNFβ with high affinity. These receptors are expressed by most cell types; circulating soluble forms of both receptor types have been detected in serum and urine, and may function as systemic TNF antagonists.

TNFα has been shown to be operative in the pathophysical processes of numerous chronic and acute diseases. Pretreatment of mice and primates with anti-TNFα antibodies induces tolerance of otherwise lethal doses of endotoxin or bacteria. Also, patients with septic shock who received TNFα antibodies displayed improvements in cardiac function. The development of TNFα antagonists, including soluble TNFR and anti-TNF antibodies, may therefore lead to more effective therapies for the management of septic shock and other inflammatory diseases. The use of TNFα as an anti-neoplastic agent has not yielded similar successes; cancer patients treated with TNFα exhibited numerous toxic side effects, reminiscent of high dose IL-2 therapy. Since TNFα appears to be beneficial at the local level in a variety of systems, this cytokine may prove useful pending the development of targeted delivery systems.

12. References

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13. Related products

Human cytokine ELISA systems from the Biotrak assay range

Unlabelled and radiolabelled growth factors and cytokines

Cell proliferation assay system and reagents

Cell proliferation kit (for immunocytochemical/ immunohistochemical measurement)	RPN 20
Monoclonal anti-bromodeoxyuridine	RPN 202
Cell proliferation labelling reagent	RPN 201

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RPN2718PL2 Rev E 04/2008