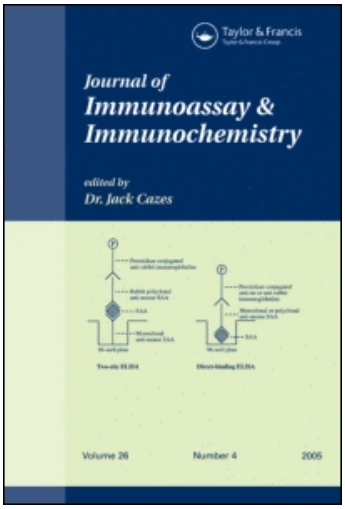


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# The Optimisation of a Murine Tnf-α Elisa and the Application of the Method to other Murine Cytokines

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# THE OPTIMISATION OF A MURINE TNF-α ELISA AND THE APPLICATION OF THE METHOD TO OTHER MURINE CYTOKINES

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## ABSTRACT

Cytokines occur in biological systems at low levels of concentration, therefore assays developed to measure them must be very sensitive. Enzyme linked immunosorbent assays (ELISA's) developed using manufacturers recommended end points can detect cytokines to picogram levels but the lower parts of their standard curves can be unreliable. In this study the relative merits of different substrate systems - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2 forms of tetramethyl benzidine (TMB), were investigated with regard to assay sensitivity. Further, a signal amplification method involving biotinylated tyramine has been used to increase the absorbance signal and thus the assay sensitivity and to achieve a sigmoidal standard curve. The amplified assay approach has been applied successfully to achieve more sensitive detection of TNF-α and improve the sensitivity of assays for a wide range of other cytokines. The optimised amplification method is the same for all the cytokine ELISA's performed in this work and this enables them to be performed simultaneously to allow multiple cytokine analysis of one sample. (KEY WORDS: ELISA, substrate, amplification, cytokines.)

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# INTRODUCTION

TNF-α is a cytokine produced primarily by macrophages and lymphocytes in response to a wide variety of stimuli including mitogens, other cytokines, bacteria, viruses and parasites. It is a 17 kDa protein that exists as a trimer with a molecular weight of 50,000 under native conditions. The main target cells for this pro-inflammatory cytokine are fibroblasts and endothelial cells where it initiates inflammation, catabolism (cachexia), fibrosis and the production of other cytokines (IL-1, IL-6 and GM-CSF) and adhesion molecules. TNF-α has anti-tumour activity and plays a role in immune modulation, viral replication, haematopoiesis, inflammation, anorexia, septic shock, infection and immunity (1).



Many researchers now wish to measure cytokines to gain an insight into immune response mechanisms (2,3). Such information could be used to investigate the pathogenesis of disease and the nature of cellular activation (4). Potentially cytokines could be used in therapy, or as messengers to be either induced by other cytokines or blocked by antibodies.

Since the measurement of cytokine levels is applicable to biological systems, the target protein can be present at very low levels ($10^{-9}$ g) which means that the assay needs to be very sensitive (5).

In this study we have used commercially available antibodies to develop assays with increased absorbance signal and sensitivity. Initial investigations involved the trial of different substrate systems to increase the colour produced, but amplification

of the signal with biotinylated tyramine (6,7) achieved the effect of increased assay sensitivity.

## MATERIALS AND METHODS

### Reagents

Unless otherwise stated all chemicals were purchased from Sigma, Poole, UK. Murine TNF-α, IFN-γ, IL-2, IL-4 and IL-12 antibodies and recombinant proteins were obtained from Genzyme Diagnostics, West Malling, UK; IL-10 and GM-CSF antibodies and recombinant proteins were obtained from Becton Dickinson UK Ltd, Oxford; UK. IL-6 antibodies and recombinant proteins were obtained from R&D Systems Europe Ltd, Oxford, UK; Kirkegaard and Perry Laboratories (KPL) TMB (tetramethyl benzidine) Microwell Peroxidase Substrate System was purchased from Dynex technologies Ltd, West Sussex, UK. Sulphuric acid was obtained from British Drug House, UK. Immulon II ELISA plates were obtained from Dynatech, Billinghurst, UK. Tween 20, Dulbecco's Modified Eagle's Medium (DMEM) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Company Ltd, Poole, Dorset, UK; Phosphate bufferd saline (PBS) was obtained in tablet form from Oxoid, Basingstoke, Hampshire, UK; The water used was deionized.

### Comparison of Enzyme Substrates

The methods used employ different substrates for the enzyme horseradish peroxidase (HRPO), so that when hydrogen peroxide is supplied to the reaction mixture each substrate indicates a chemical change in a quantitative way.

The fully optimised TNF-α assays were carried out as follows:- ELISA plates were coated with 100 µl hamster anti-mouse TNF-α at a concentration of 6 µg/ml overnight at 4 $^0$C in phosphate buffered saline. The plate was washed three times with 300 µl/well of PBS containing 0.02% Tween 20 (PBST). The wells were blocked with 200 µl of 1% BSA in PBS for two hours at 37 $^0$C. The plate was washed again as above and recombinant mouse TNF-α standards ranging from 1000 pg/ml to 15.625 pg/ml and test controls all diluted in 50% PBST / 50% DMEM were added (100 µl/well). The plate was incubated at 37 $^0$C for two hours. After washing as above, goat anti-mouse TNF-α HRPO diluted to 3 µg/ml in PBST containing 1% BSA was added (100 µl/well) and the plate incubated at room temperature for thirty minutes. The plate was washed again as above and the substrate added.



Three different substrates were trialled at 100 µl/well at room temperature:

a) ABTS: Three x 10 mg ABTS tablets were dissolved in 50 ml 0.088M phosphate / 0.056M citrate buffer pH 4.4 containing 5 µl 30% hydrogen peroxide solution; the colour reaction was left to proceed for 20 minutes before reading the absorbance at 414 nm.

b) TMB: TMB-2HCl 2 x 1 mg tablets were dissolved in 100 ml 0.05M phosphate/citrate/urea/hydrogen peroxide buffer pH 5.0. The colour reaction was stopped with 2M sulphuric acid after 15 minutes and the absorbance read at 450 nm.

c) Kirkegaard and Perry Laboratories (KPL) TMB Microwell Peroxidase Substrate System: Prepared according to manufacturers instructions, the reaction was allowed to

proceed for 5 minutes before stopping with 2M sulphuric acid and reading the absorbance at 450 nm.

Amplification

This method employs horseradish peroxidase label to catalyse the deposition of biotin in the assay. The deposited biotin is then reacted with streptavadin-labelled horseradish peroxidase, thereby resulting in the deposition of more enzyme to catalyse the appropriate substrate.

Tyramine (40 mg) was dissolved in 1 ml dimethyl sulfoxide (DMSO) and 100 mg N-Hydroxysuccinimidobiotin was dissolved in a further 1 ml DMSO. The two solutions were mixed together and incubated overnight with gentle mixing at room temperature to form the biotinylated tyramine (BT). The reacted mixture was aliquoted into small amounts and frozen at -20 $^{0}$C.

The amplification mixture was made as follows: 0.6 µl of BT was added per ml of 50mM Tris/HCl pH 8.0 buffer containing 0.01% hydrogen peroxide.

The ELISA was performed as before and the curve amplified after the HRPO stage. The amplification mixture, 100 µl/well, was added to the plate and incubated for 15 minutes at room temperature.

The plate was washed three times with PBST (300 µl/well) and ExtrAvidin peroxidase diluted x1000 in 1% w/v Bovine serum albumin in PBS was added (100 µl/well) to the plate and incubated at room temperature for 15 minutes. After washing

the plate, the ABTS substrate was added (100 µl/well). The OD was read at 414nm after 5 minutes.

Assay Validation

The amplified assay was tested to ensure that it was accurate and reproducible by the following methods:

Recovery

Recombinant mouse TNF-α was diluted to make two concentrations. One was used both for the assay standards and to provide increasing amounts of known concentration. The other was assayed and used as a 'spike' for detection in the known increasing concentrations. All test concentations were assayed in quadruplicate This method measures assay accuracy as it tests whether the 'spike' amount is measured accurately when added to the larger amounts.



Inter assay variability

Three different concentrations of TNF-α were tested in quadruplicate against the standard curve in six consecutive assays, performed on different days. The standards and test concentrations were diluted freshly on the day of assay performance. The aim of this experiment was to measure the reproducibility of the test sample results from assay to assay. The coefficient of variation was calculated by dividing the pooled standard deviation by the pooled mean and has been presented as a percentage after multiplying by one hundred (%CV) (8).

Intra assay variability

In order to measure variability within each assay, four test controls were arranged in duplicate wells so that each test control appeared on each row but in different columns (9). This experiment determines whether the sample results are affected by their position on the plate.

Amplification of other cytokine ELISA's

The amplification system was applied after the HRPO stage to other murine cytokine ELISA's performed in our laboratory, including IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ and GM-CSF.



## RESULTS

The Effects of Different Substrates on the ELISA.

When the assay was performed using ABTS and Sigma TMB as substrate, the colour yielded was poor with very small changes in absorbance for large changes in concentration (Figure 1).

The use of the KPL Substrate System greatly improved the absorbance values for the TNF-α standards, but the curve obtained was linear with no plateau, rather than sigmoidal. The high TNF-α standard concentrations gave absorbencies which were very close to the limit of the accurate measurable range of the spectrophotometer.

The Effect of Amplification on the ELISA.

With the amplified assay (Figure 1), the top TNF-α standard concentration could

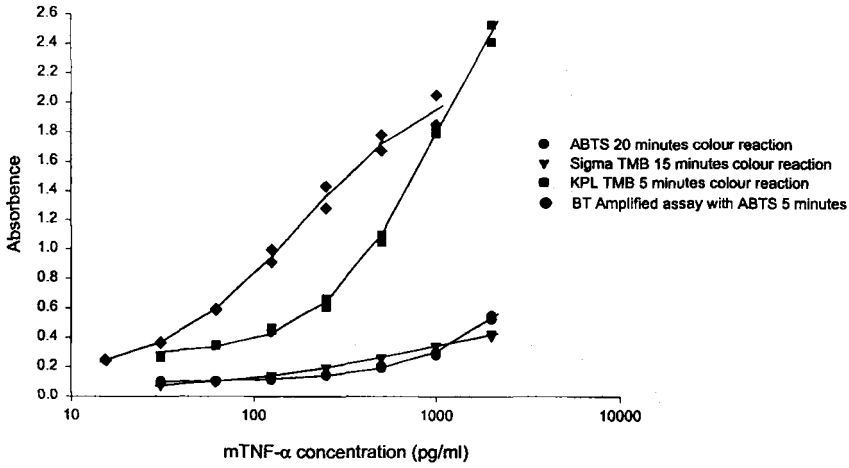


Figure 1. The effects of different substrates and BT amplification on the Genzyme mTNF-α ELISA. Averaged blank values were: ABTS 0.095, Sigma TMB 0.063, KPL TMB 0.198 and BT 0.182. All measurements were made in duplicate and each individual well has been plotted.



be decreased to achieve the sigmoidal curve that was well within the accurate measurable range of the spectrophotometer.

Validation of the Amplified ELISA

The range of the standard curve was 1000 to 15.6 pg/ml mouse TNF-α and the standard curves were reproducible when plotted over a 9 month period (Figure 2).

The assay showed good recovery of mouse TNF-α (Table 1) with all results falling within +/- 5% of the spiked amount. These results demonstrated the assay to be accurate.

The inter assay variability was within acceptable limits, ranging from 5.78 to 9.72

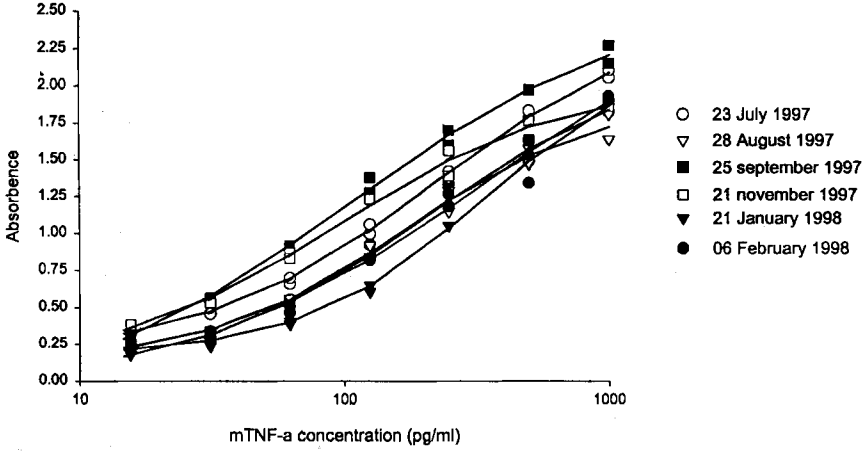


Figure 2. A curve comparison for the mTNF-a ELISA over a nine month period.



TABLE 1

Recovery of Mouse TNF-α.

| Concentration mouse TNF-α added (pg/ml) [a] | % mouse TNF-α recoverd [b] |
|---|---|
| 20 | 104.2 |
| 50 | 97.0 |
| 100 | 102.5 |
| 150 | 100.65 |
| 300 | 99.2 |

[a] Known amounts of TNF-α were added to two different samples containing low quantities of TNF-α to determine recoveries.
[b] % Recoveries are averages from two assays using four wells per assay for each concentration.

TABLE 2

Inter Assay Variability.

| | Low concentration | Middle concentration | High concentration |
|---|---|---|---|
| No. of assays | 6 | 6 | 6 |
| Mean (pg/ml) | 75.67 | 152.5 | 320.5 |
| SD | 5.75 | 8.82 | 31.14 |
| %CV | 7.59 | 5.78 | 9.72 |

TABLE 3

Intra Assay Variability.

| | Concentration 1 | Concentration 2 | Concentration 3 | Concentration 4 |
|---|---|---|---|---|
| No. of wells | 20 | 20 | 20 | 20 |
| Mean (pg/ml) | 44.5 | 84.5 | 157.5 | 304 |
| SD | 3 | 6 | 13.6 | 31 |
| %CV | 6.7 | 7.1 | 8.6 | 10.2 |

TABLE 4

The Standard Curve Ranges for the Amplified Cytokine ELISA's.

| Cytokine | Lowest standard (pg/ml) | Highest Standard (pg/ml) |
|---|---|---|
| mTNF-α | 15.6 | 1000 |
| mIFN-γ | 11.7 | 750 |
| mIL-2 | 7.8 | 500 |
| mIL-4 | 7.8 | 500 |
| mIL-6 | 31.3 | 2000 |
| mIL-10 | 39.1 | 2500 |
| mIL-12 | 15.6 | 1000 |
| mGM-CSF | 31.3 | 2000 |



%CV (Table 2). The %CV results show that there is minimal drift in the assay when the same concentration test controls were assayed on different days.

The intra assay variability (Table 3) gave results from 6.7 to 10.2 %CV. This demonstrates that the position of the test controls on the 96 well plate had little effect on the final result.

Amplification of other cytokine ELISA's

The standard curve ranges obtained while achieving sigmoidal curves can be seen in Table 4. The highest standard required is 2500 pg/ml for the IL-10 ELISA and the

lowest is 500 pg/ml for the IL-2 and IL-4 assays. This was achieved using biotinylated tyramine and HRPO at the same concentrations for all the cytokine ELISA's to enable them to be easily performed at the same time.

## DISCUSSION

The absorbance achieved with the TMB and ABTS substrates showed that they were not suitable for the TNF-α ELISA. The absorbance was increased by using the KPL substrate system but the shape of the standard curve was still not sigmoidal. To achieve a true sigmoidal curve the concentration of the top standard would have to have been raised. The top standard was nearing the limit of the accurate measurable range for the spectrophotometer so this was not desirable.

The BT amplification made the ELISA more sensitive, shifting the curve to the left and the absorbance values were well within the measurable range. In the amplified assay the curve assumed a sigmoidal shape with a plateau appearing, so that the amplified assay had increased sensitivity and a wide accurate working range. Being more sensitive, the assay is better for measuring samples of low concentration, so that the need for sample extraction and concentration is reduced. Samples at higher concentrations may be diluted for assay so that less sample is needed and interfering substances can be diluted out.

All the validation results for the amplified assay fell within 10.2 %CV. These results are highly acceptable considering that the figures obtained for the inter assay variation also contain a dilution error since it was not possible to store the more dilute test concentrations.

Because of their heat labile nature, cytokines are difficult to measure (10). Samples cannot be repeatedly re-frozen and thawed without losses (3). It is important that the ELISA's to measure them are reliable and able to be performed at the same time to allow one sample to be assayed for several different cytokines simultaneously. These aspects are particularly important when measuring cytokine levels in supernatants from T cell proliferation assays. These samples are small, many are irreplaceable since they are the end point of a long running experiment and they need to be assayed for several different cytokines at the same time.



The advantage of this amplification method is that the curve produced is sigmoidal which means that the sample results will be calculated using the linear portion of the curve. Assays from other sources often do not give sigmoidal curves on a log:lin plot. This means that the sample results are calculated using only the lower portion of the standard curve which could be less accurate. With a standard curve range of 1000 to 15.6 pg/ml and a sensitivity of 3.2 pg/ml this assay compares favourably with that of other murine TNF-α assays. Commercially available murine TNF-α assays have standard curve ranges from 2240 to 35 pg/ml, 2000 to 43 pg/ml, 1500 to 23.4 pg/ml with sensitivities quoted as 15 pg/ml, 10 pg/ml and <5.1 pg/ml respectively.

Since the binding of biotinylated tyramine is catalysed by HRPO this amplification system may be applied to any assay system which contains an HRPO step. It is cost-effective as it does not require the purchase of expensive antibody conjugates. The biotinylation of the tyramine is reproducible and an easy technique to perform.

The advantages of increased sensitivity gained should be considered against the

slightly increased assay time required for the extra incubation periods and plate washing which this method requires.

This amplification method is applicable to those ELISA systems utilizing HRPO for highly sensitive measurement of cytokines and other analytes present at low concentrations.

## ACKNOWLEDGEMENT

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