TGFα- ELISA Kit
Cat. No. QIA61

Note that this user protocol is not lot-specific and is representative of the current specifications for this product. Please consult the vial label and the certificate of analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions. Full details are available at www.calbiochem.com.

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Storage

Upon receipt, **standards must be stored at –20°C.** All other components may be stored at –20°C or 4°C (do not refreeze after thawing). Do not expose reagents to excessive light. Allow kit reagents to warm to room temperature before use.

Intended Use

The Calbiochem® TGFα ELISA is a non-isotopic immunoassay for the *in vitro* quantitation of human Transforming Growth Factor Alpha (TGFα) in serum, tissue culture media, and other fluids.

Background

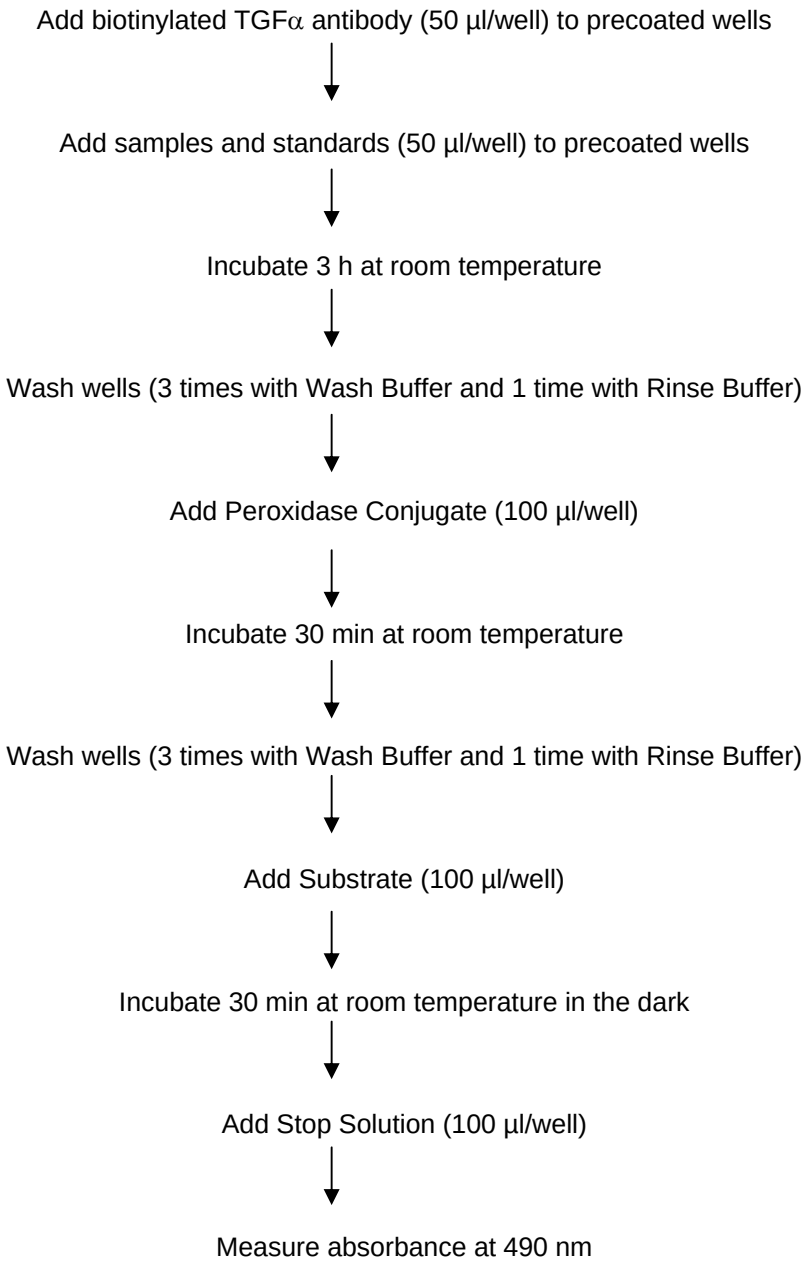
Transforming growth factors (TGFs) are potent mitogenic polypeptides. Two classes of TGFs have been identified: TGFα and TGFβ. TGFα is synthesized as a 160 amino acid, transmembrane precursor. The precursor undergoes sequential external proteolytic cleavage, releasing TGFα species ranging in molecular weight from 6-25 kDa. Mature TGFα is an acid- and heat-stable 50 amino acid polypeptide of 5.5 kDa . It is secreted by a variety of transformed cells and tumors, and by a number of embryonic cells as well as some normal adult tissues including the brain and skin keratinocytes. Mature TGFα is approximately 30% homologous, at the amino acid level, to epidermal growth factor (EGF). TGFα binds to the EGF receptor, mediates tyrosine phosphorylation of the receptor, and promotes anchorage-independent growth of normal rat fibroblasts in soft agar in the presence of transforming growth factor-beta. Transformation of cells, *in vitro*, by certain viruses including SV40 and polyoma virus, as well as transformation by the activated ras oncogene can lead to increased secretion of TGFα. TGFα may play a role in tumor formation by an autocrine/paracrine mechanism whereby TGFα promotes and sustains the transformed character of the cells from which it is secreted as well as that of neighboring cells. The biological relevance of TGFα in tumor development is supported by reports that multiple molecular weight forms of TGFα may be found in the urine of cancer patients in amounts which differ from those found in normal urine samples. TGFα may also play a role in normal development including wound healing. The major difficulty impeding the investigation of the normal and pathological roles of TGFα *in vivo* has been the use of activity-based biological assays that do not distinguish between TGFα and other related molecules (e.g., EGF). The Calbiochem® TGFα ELISA is highly specific and can detect less than 10 pg/ml of human TGFα in a variety of biological fluids and tissue culture media.

Principle of the Assay

The Calbiochem® TGFα ELISA is a “sandwich” enzyme immunoassay which utilizes affinity purified goat polyclonal antibodies specific for mammalian TGFα. Polyclonal TGFα antibodies have been immobilized onto the surface of the plastic wells provided in the kit. The sample to be assayed and biotinylated goat TGFα antibodies, are added to the wells, and allowed to incubate for a period of time. During this time, any TGFα present in the sample binds to both the capture antibody on the plate, and to the reporter antibody (biotinylated) in solution. Unbound material is washed away. The reporter antibody is bound, in turn, by streptavidin-horseradish peroxidase.

The horseradish peroxidase catalyzes the conversion of the chromogenic substrate o-phenylenediamine dihydrochloride from a colorless solution to a light amber solution, the intensity of which is proportional to the amount of TGFα bound to the plate. After stopping further color development, the colored reaction product is measured using a spectrophotometer. Quantitation is achieved by the construction of a standard curve using known concentrations of TGFα. By comparing the absorbance obtained from a sample containing an unknown amount of TGFα with that obtained from the standards, the concentration of TGFα in the sample can be determined.

Summary of Procedure



Materials Provided

Standards should be assayed in duplicate. A standard curve must be performed on the same plate and at the same time as the samples. The TGFα ELISA provides sufficient reagents to run two sets of standard curves, and 41 samples (if assayed in duplicate all at once using one standard curve), or 34 samples (if assayed on two separate occasions using two standard curves).

- **TGF alpha Polyclonal Antibody Coated Plate:** 96 removable wells coated with TGFα polyclonal antibody.
- **TGFα Standards:** two vials containing lyophilized, synthetic, biologically active TGFα.
  - *Reconstituted standards should be discarded after one use.*

- **Plate Sealers:** Plate sealers are provided to cover the plate during incubations.
- **Reporter Antibody:** (500 µl) 20X concentrated solution of biotinylated goat TGFα antibody.
- **Peroxidase Conjugate:** (200 µl) 100X concentrated solution of streptavidin conjugated to horseradish peroxidase.
- **Peroxidase Substrate:** (5 tablets) *O*-Phenylenediamine (OPD) and (20 ml) Substrate Diluent.
- **Stop Solution:** (20 ml) 2.5 sulfuric acid.
- **Rinse Buffer:** (30 ml) 10X concentrated buffer containing bovine serum albumin, a surfactant and 0.1% 2-chloroacetamide.
- **Antigen Extraction Agent (AEA):** Use as directed in sample preparation section for the extraction of TGFα from cell preparations
- **Assay Buffer:** (30 ml) Buffer containing bovine serum albumin, a surfactant, protein stabilizers and 0.1% 2-chloroacetamide.

NOTE: The amount of Assay Buffer provided is sufficient when used as directed. Use only as much Assay Buffer as needed to ensure delivery of 100 µl of sample to each well. If a large excess of diluent sample is prepared (e.g., 1 ml rather than 0.22 ml needed for each sample, in duplicate) Assay Buffer may become limiting.

- **Wash Buffer:** (100 ml) 20X concentrated buffer and surfactant.

Materials Required but not Provided

- 2-20 µl, 20-200 µl and 200-1000 µl precision pipetters with disposable tips.
- Wash bottle or multichannel dispenser for washing.
- Microcentrifuge tubes or other appropriate containers for mixing low volumes.
- 1 liter graduated cylinder.
- Deionized H_2O.
- Plastic wrap.
- Adhesive tape.
- Spectrophotometer capable of measuring absorbance in 96-well plates at a wavelength of 490 nm. Use of a 630 nm reference beam is suggested.
- Disposable paper towels.
- 2 µm syringe filter and syringe.

Precautions and Recommendations

- Store **standards at –20°C.** All other components may be stored at –20°C or 4°C (do not refreeze after thawing). Do not expose reagents to excessive light. Warm kit reagents to room temperature before use (let sit at room temperature approximately 30 min before use.)
- Use only the wells provided with the kit.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain 2-chloroacetamide. Care should be taken to prevent direct contact with these products.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Dispose of samples properly.
- Do not handle the substrate tablets with fingers or permit contact with skin, metal or oxidizing agents. Dispose of OPD (*o*-phenylenediamine) containing solutions in compliance with local regulations.
- Wear disposable gloves and eye protection when handling Stop Solution (2.5N sulfuric acid).

Sample Preparation

Cell Lysate Protocol. Numerous extraction protocols can be used. The following protocol has been shown to work with a number of cell lines. It is provided as an example of a suitable extraction procedure, but should not be construed as necessarily being the method of choice. Users may wish to experiment with extraction procedures that work best in their hands.

1. For suspension cells, pellet by centrifugation, remove supernatant, resuspend with PBS and pellet by centrifugation. For attached cells, remove supernatant from cells (you may save the supernatant for testing in the ELISA). Wash cells once with PBS, harvest cells by scraping and gentle centrifugation.
2. Aspirate PBS leaving an intact cell pellet (at this point the cell pellet can be frozen at –80°C and lysed at a later date). We recommend for every 5 x 10^6 cells, resuspend the pellet in 1 ml of Resuspension Buffer (50 mM Tris containing 5 mM EDTA, 0.2 mM PMSF, 1 ug/ml pepstatin, and 0.5 ug/ml of leupeptin adjusted to pH 7.4.
3. Add 20 µl of Antigen Extraction Agent (AEA provided) for every 100 µl of cell suspension.
4. Incubate 30 min on ice with occasional vortexing.
5. Transfer extracts to microcentrifuge tubes and centrifuge for 5 min at 500 x g at 4°C.
6. Aliquot cleared lysate to clean microcentrifuge tubes. The sample should be aliquotted to avoid multiple freeze/thaws. These samples are now ready for analysis according to the instructions provided in the Detailed Protocol. Samples may be stored at –20°C for future testing in the TGFα ELISA.
7. Samples found to contain greater than 1000 pg/ml TGFα (i.e., outside the range of the standard curve) must be diluted with Assay Buffer (provided), so that the TGFα concentration falls within the range spanned by the standard curve, and assayed again.
8. Appropriate negative controls should be included. For example, if TGFα is being measured in cell conditioned medium, a sample of the same medium which has not been exposed to cells should also be assayed.
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Detailed Protocol

The TGFα ELISA is provided with removable strips of wells so the assay can be carried out on separate occasions. **Since conditions may vary, a standard curve must be determined each time the assay is performed.** Both standards and samples should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross contamination of reagents or samples.

1. Remove the appropriate number of wells from the foil pouch. Return any unused wells to the foil pouch containing the desiccant pack. SEAL TIGHTLY and store at 4°C. Let all other kit components sit at room temperature until used. *Best results will be obtained using reagents at room temperature.*
2. Prepare a working solution (1X) of Rinse Buffer by adding 30 ml of 10X concentrated solution to 270 ml of deionized water. Mix well.
3. Prepare a working solution (1X) of Wash Buffer by adding 50 ml of the 20X concentrated solution to 950 ml of deionized water. Mix well.
4. Reconstitute Lyophilized Standard (refer to vial label for lot specific reconstitution volume of dH_2O and/or Assay Buffer) to yield a stock solution of 1000 pg/ml. Let the reconstituted standard sit for 15 min, with occasional swirling. Avoid excessive agitation of the standard. Serial dilutions of the standard should be made with Assay Buffer. To make serial dilutions of the standard, obtain 6 tubes and label them, 1000, 500, 250, 125, 62.5, 0 pg/ml. Add 250 µl of Assay Buffer into each tube, except the 1000 pg/ml tube (first tube) which gets “undiluted” standard. Remove 500 µl from the undiluted, reconstituted standard vial (1000 pg/ml) and add it to the first tube. Remove 250 µl from the first tube (1000 pg/ml) and add it to the second tube (500 pg/ml) and mix gently. Repeat this procedure until you reach the fifth tube (62.5 pg/ml). The last tube (0 pg/ml) should contain only 250 µl of Assay Buffer.

5. Dilute Reporter Antibody 1:20 in Assay Buffer to provide 55 µl of 1X solution for each of the sample and standard wells. (For example: add 60 µl to 1,140 µl of Assay Buffer to make 1.2 ml of working solution, sufficient for 20-21 wells).
6. Add 50 µl of diluted Reporter Antibody to appropriate wells, in DUPLICATE.
7. Add 50 µl of standards/samples to appropriate wells, in duplicate, on top of reporter antibody.
8. Cover plate with a plate sealer and incubate at room temperature for 3 h.
9. Dilute a sufficient amount of the Peroxidase Conjugate 1:100 in Assay Buffer to provide 100 µl of 1X solution for each of the sample and standard wells. (For example: add 11 µl to 1,089 µl of Assay Buffer to make 1.1 ml of working solution, sufficient for 10 wells). Filter with a 0.2 µm syringe filter prior to use.
10. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
11. Wash wells one time using 200 µl of 1X Rinse Buffer per well. *Do not let the Rinse Buffer incubate on the plate for more than five min.* Empty the contents of the wells by inverting the plate over a sink then tapping dry on paper towels.
12. Add 100 µl of the 1X Peroxidase Conjugate into each well. Cover with plastic wrap and incubate 30 min at room temperature.
13. Prepare sufficient Substrate Solution to provide 100 µl for each well. Substrate Solution is prepared by dissolving one (1) OPD tablet per 4 ml of Substrate Diluent and mixing gently. Once prepared, substrate solution should be used within 30 min. AVOID EXPOSURE TO LIGHT.

    WARNING: Do not handle substrate tablets with fingers or permit contact with skin, metal or oxidizing agents. If contacted, flush with water. Solutions should be disposed of in compliance with local regulations.
14. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
15. Wash wells one time using 200 µl of 1X Rinse Buffer per well*. Do not let the Rinse Buffer incubate on the plate for more than five min*. Empty the contents of the wells by inverting the plate over a sink then tapping dry on paper towels.
16. Add 100 µl of Substrate Solution to each well and incubate IN THE DARK at room temperature for 30 min.
17. Add 100 µl of Stop Solution to each well in the same order as the previously added Substrate Solution.
18. Measure absorbance in each well using a spectrophotometric plate reader at a wavelength of 490 nm. Wells must be read within 30 min of adding the Stop Solution.

Evaluation of Results

1. Average the duplicate absorbance values for each standard, including the zero, and all sample values.
2. On graph paper, plot the mean absorbance values for each of the standards on the Y axis, versus the concentration of each standard (pg/ml) on the X axis.
3. Determine the concentration of unknowns by interpolation from the standard curve. There are a variety of plate reader software packages available (Softmax, Molecular Devices Corporation, Menlo Park, CA; KinetiCalc, BioTek Instruments, Inc. Winooski, VT) for analysis of plate data, which simplifies this process.
4. For samples which have been diluted, the TGFα concentration must be multiplied by the dilution factor (ie., if the sample was diluted five-fold, then the TGFα value obtained from the standard curve must be multiplied by five).

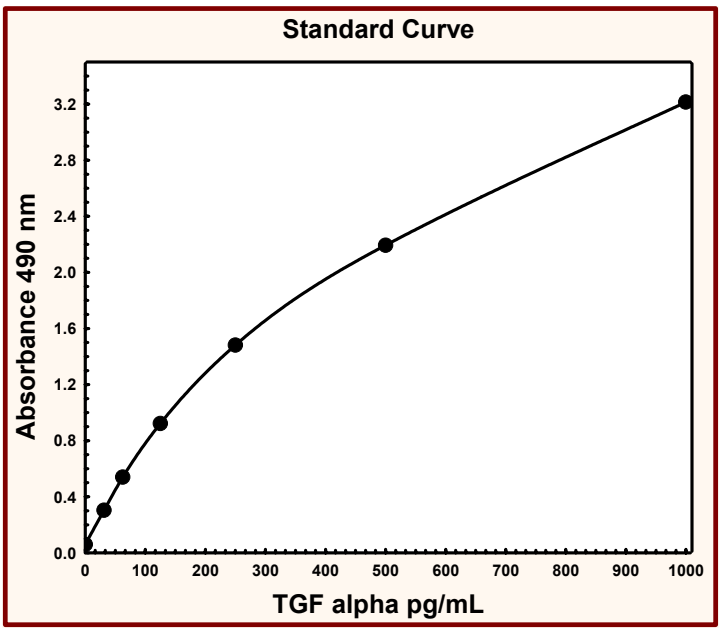

Figure 1. Standard Curve. The mean signal of each standard run in replicates of three in eight assays using two different lots of plates and two different lots of detector antibody.

Assay Characteristics

A. Sensitivity

Studies have demonstrated that TGFα ELISA can distinguish 2.1 pg/ml from zero with a 95% confidence, therefor the ELISA can easly distinguish 6 pg/ml from zero.

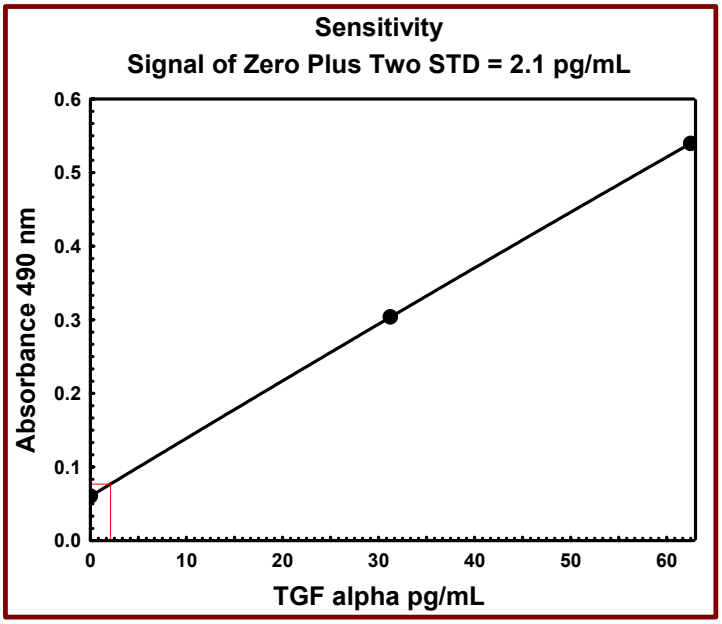

Figure 2. Sensitivity. The lower limit of detection (LLD), commonly used to define sensitivity, was measured by assaying four replicates of zero eight times using two different lots of plates and two different lots of detector antibody. The grand mean signal and pooled standard deviation of zero was calculated. The grand mean of each standard (run in replicates of three in the eight assays) was used for the standard curve (Figure 3), and the response, mean signal of zero plus two standard deviations, read in dose from the standard curve is the LLD; that is, the smallest dose that is not zero with 95% confidence.

B. Specificity

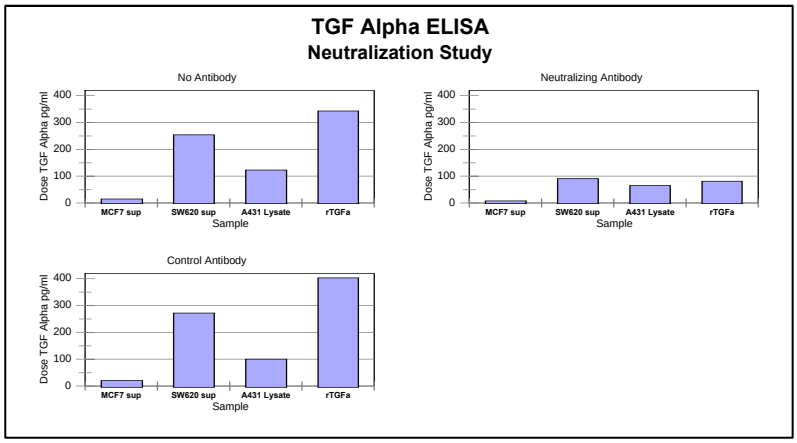

Figure 3. Levels of TGFα in cell culture supernatants, cellular lysates and recombinant protein detected by the ELISA after immunoaffinity extraction of TGFα by an antibody that is not used in the ELISA.

The TGFα ELISA detects soluble and intracellular TGFα. Specificity was demonstrated by immunoaffinity extraction (inhibition of assay signal) of TGFα positive samples by a specific TGFα antibody. The TGFα antibody, which is not a component of the ELISA, extracted almost all TGFα (almost the entire signal was lost), while the control antibody (non-TGFα antibody) did not affect the signal of the TGFα positive samples (see Figure 2). The non-ELISA TGFα antibody extracts the same portion of the TGFα activity detected by this immunoassay from the biological samples as it did from a recombinant TGFα sample demonstrating that the assay is specificity for the soluble and intracellular TGFα (Figure 2).

No measurable cross-reactivity was observed when human Epidermal Growth Factor (hEGF) and amphiregulin were assayed at a concentration greater of 1000 pg/ml.

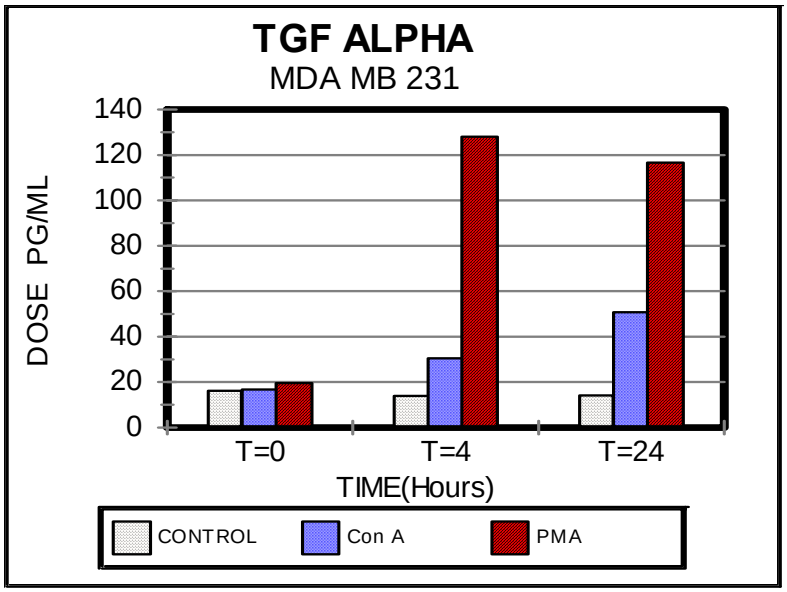

Figure 4. Phorbol ester and Concanavalin A induced up-regulation of TGFα in MDA MB 231 cells. TGFα is synthesized and transported to the cell surface as a membrane-anchored precursor (proTGFα) that is converted to the soluble form by proteolytic cleavage. ProTGFα cleavage is activated in response to tumor-promoting phorbol ester. Concanavalin A is a mitogen known to upregulate cytokine production in numerous cell lines.

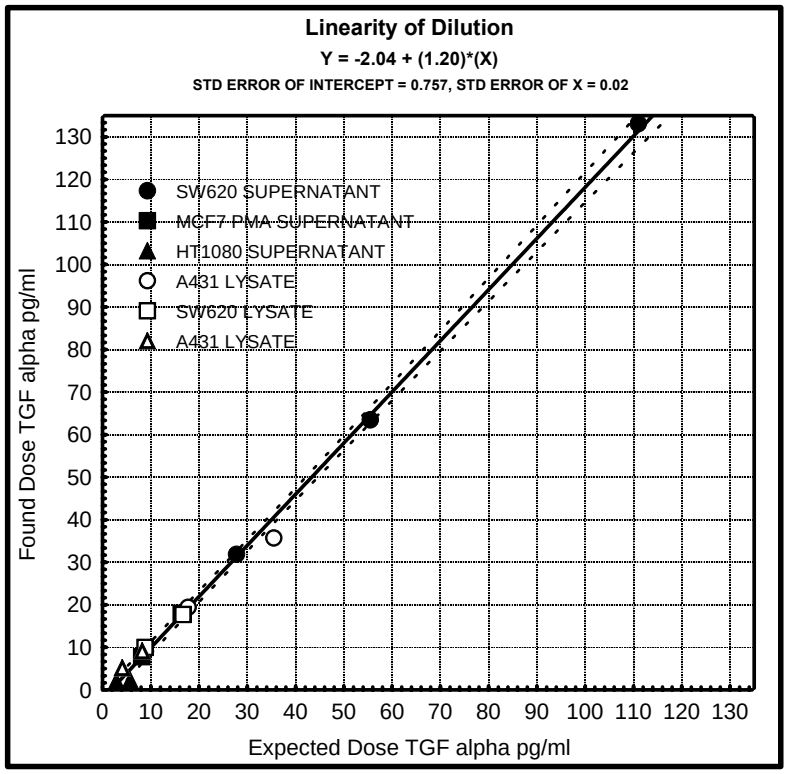

Figure 5. Parallelism: The study tested dilution-recovery of three positive supernatant and three lyste samples. The dilutions were run in the TGFα ELISA and the "found" doses were plotted against the "expected" doses to determine linearity of dilution. The slope is close to one and the intercept is not significantly different from zero. These studies are consistent with the absence of cross-reacting and matrix effects such as pH, salts, and endogenous binders that interfere with the reagents used in the assay.

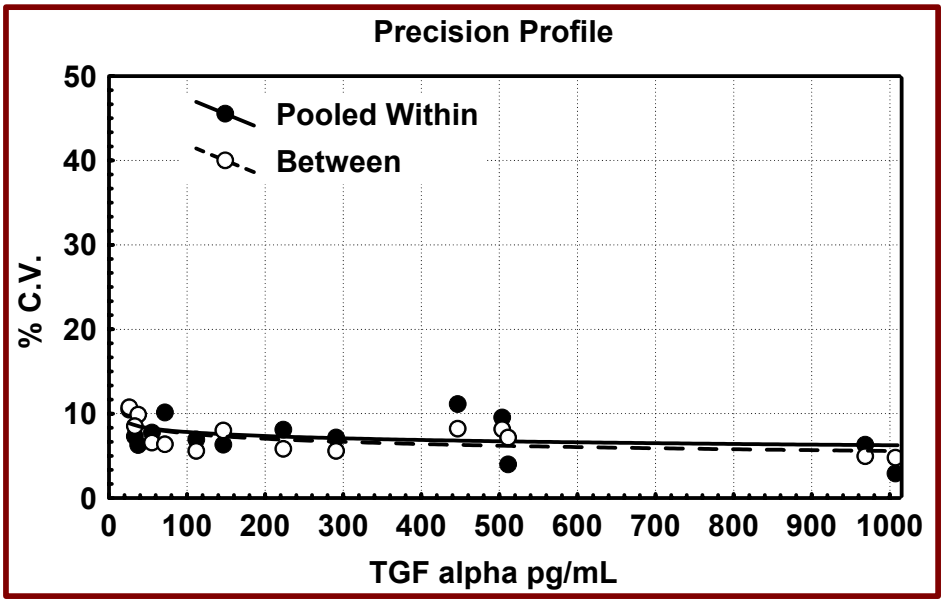

Figure 6. Precision. The pooled coefficients of variation (according to the formula of Henry et. al., 1974) and between assay coefficients of variation are plotted against PCNA levels. The pooled data were collected from samples run eight times using two different lots of plates and two different lots of detector antibody in replicates of three.

Recovery

Human TGFα (900 pg/ml) was added to each of five (5) undiluted normal human sera (NHS) and five (5) undiluted normal human plasma (NHP). The spiked samples were analyzed neat (undiluted) and diluted 1:2, 1:4 and 1:8 with Assay Buffer. Results are expressed as a percent of the expected value +/- one standard deviation.

% of Expected Value				
Sample	**Neat**	**Diluted 1:2**	**Diluted 1:4**	**Diluted 1:8**
NHP	**47+/-18**	**72+/-20**	**62+/-19**	**72+/-17**
NHS	**47+/-20**	**54+/-39**	**38+/-28**	**32+/-24**

Reagent Stability

All of the reagents included with the TGFα ELISA have been tested for stability. Assay Buffer should not be used if cloudiness or solid matter is present. Coated plates should be stored at 4°C in the original foil bag containing a desiccant pack.

References

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