

Ultrasensitive ELISA for Measurement of Human Cytokine Responses in Primary Culture

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Abstract

ELISAs offer excellent specificity and, once fully optimized, sensitivity that rivals that of bioassays. The major variables that need to be experimentally determined when developing an ELISA are the optimal number of fresh cells required per well, the optimal antigen concentrations for stimulation, period of culture, and the anticipated intensity of the response. In this chapter, we review the major factors to be considered in the development and application of ultrasensitive ELISAs to the analysis of human immune responses. We specify the conditions we have found to be optimal for quantifying a number of cytokines of demonstrated relevance to human immune regulation and discuss the major pitfalls inherent in this approach.

Key Words: Cytokine; ELISA; primary culture; antigen-specific; human.

1. Introduction

Over the last 15 yr, there has been a shift from reliance on assessment of human serum Ig levels as indicators of immune status to analysis of human cytokine production and mechanisms of immune regulation. The major approaches taken include (i) derivation of human T-cell lines and clones, (ii) polyclonal stimulation of freshly derived lymphocytes directly *ex vivo*, (iii) short-term antigen-stimulated primary culture, and (iv) analysis of mRNA synthesis by RT-PCR and/or *in situ* hybridization. Each of these approaches is considered in detail elsewhere in this volume.

Experimental approaches available for quantitation of human cytokine protein levels have evolved from reliance on bioassays (which have superb sensitivity but are inherently more variable from assay to assay and have the potential for

From: *Methods in Molecular Medicine: Allergy Methods and Protocols*
Edited by: M. G. Jones and P. Lympny © Humana Press Inc., Totowa, NJ

specificity problems) to ELISA. ELISAs offer excellent specificity and, once fully optimized, sensitivity that rivals that of bioassays. In this contribution, we review the major factors to be considered in the development and application of ultrasensitive ELISAs to the analysis of human immune responses. We specify the conditions we have found to be optimal for quantifying a number of cytokines of demonstrated relevance to human immune regulation and discuss the major pitfalls inherent in this approach.

Whereas detailed methodology for Ag-specific stimulation is dealt with in detail elsewhere in this volume, the need for optimization of culture conditions prior to embarking on analysis must be reiterated. The major variables that need to be experimentally determined are the optimal number of fresh cells required per well, the optimal antigen concentrations for stimulation, period of culture, and the anticipated intensity of the response. Culturing an arbitrary number of cells for a single time period and then harvesting and analyzing one large lot of supernatant is convenient, but provides misleading results. Our experience has been that preliminary experiments carried out with peripheral blood mononuclear cells (PBMC) of five to seven donors usually provides a sufficiently complete picture to establish the experimental conditions required for the remainder of the study. Given that the supply of blood, hence PBMC, is invariably less than the number of experimental conditions that one wishes to investigate, a balance needs to be struck between using as few cells and little antigen as necessary while having sufficient of each to generate readily quantified, reproducible responses. In practice, we find that $2.5\text{--}5.0 \times 10^5$ PBMC/well, using a 96-well plate, is sufficient for antigens that range from "weak" (food allergens, single recombinant allergens such as Der p 1) to recall antigens that yield very strong responses (streptokinase, *Candida*).

The optimal antigen concentration needs to be determined empirically for each individual antigen, usually by testing it over a range of five half log dilutions. Fortunately, the range that stimulates strong responses is typically broad (at least one order of magnitude) and individuals do not differ dramatically in the Ag concentration that is required to yield maximal stimulation. Hence, in characterizing the cytokine response to streptokinase, one does not find that some individuals yield maximal responses at 10 pg/mL, whereas others respond best at 1000 or 50,000 pg/mL. The practical consequence of this is that once the optimal Ag concentration is determined, it can be used for all individuals in the subsequent study.

The third major variable that needs to be determined prior to undertaking a major study is the kinetics of the response. It is well established that different cytokines peak in their production at different times. Hence, in response to Ag-driven stimulation, IL-2 and IL-4 levels are generally maximal at 24–48 h whereas IFN γ and IL-13 do not reach maximal levels until day 5–day 7.

Harvesting all supernatants at, for instance, 48 h would suggest a strong IL-4 and undetectable IL-13 response whereas harvesting all supernatants at day 5 would indicate the opposite. We have found minimal differences between different individuals in the kinetics of their responses. The practical consequence is that one can establish one set of cultures to be harvested at 24 h for subsequent analysis of certain cytokines, an identical set for harvest at 48 h, and a third set for harvest at 5 d. Cytokine concentrations are then determined by the methods described below only in the relevant supernatants.

Spontaneous production of cytokine in antigen-unstimulated cultures needs to be considered. For some cytokines (i.e., IL-2, IL-4) culture in medium alone for periods of up to 10 d, well past the peak antigen-driven response, virtually never yields detectable levels. Other cytokines (notably IL-10, IL-13) are routinely detectable in control cultures at low but significant levels by days 4–6 of culture. Whereas the intensity of the antigen-stimulated cytokine production is much larger than that seen in the negative control, such cytokine production needs to be dealt with. One approach is to subtract the values obtained in control wells from antigen-stimulated wells. An alternative is to report both values independently (i.e., mean IL-13 production of 141 pg/mL in unstimulated wells vs 875 pg/mL following antigen stimulation).

Finally, we do not find significant differences in analyses of samples kept at -20 or -80°C for periods of up to 3 mo. The major concern is sublimation, which would of course dramatically alter cytokine concentrations in the remaining supernatant. This is dealt with by ensuring that the plate used to store the culture supernatants does not have large numbers of empty wells (using sterile distilled water to fill extra wells), tightly wrapping the plates with plastic wrap before storage, and prompt analysis of the cultures following completion of the experiment.

1.1. Sample Handling for Ultrasensitive ELISA

The availability of ultrasensitive ELISAs is a double-edged sword. Its sensitivity allows widespread use of physiologically relevant ligands (antigens, peptides, etc.) in the analysis of antigen-specific responses rather than pharmacologic reagents (PMA/calcium ionophore, anti-CD3, PHA) that yield intense cytokine production by virtually all T cells rather than only those specific for the nominal antigen. Given that the frequency of T cells specific for any single (nonallogeneic) antigen represents only a tiny fraction (usually $<<1\%$) of total T cells, antigen-specific stimulation is clearly a preferable method to characterize such responses. At the same time, any ultrasensitive technique is highly sensitive to experimental error. Because measuring small volumes by micropipetor is such a routine procedure, it is not always carried out with precision.

To reinforce the need for accuracy and to provide a quantifiable indicator of that individual's variability for the supervisor, we routinely have all trainees quantify the variability in their technique when first entering the laboratory.

1.2. Strategies for Enhancing Sensitivity

A few strategies are useful to further enhance sensitivity. The assays described are configured using the minimum concentrations of the reagents required in order to minimize cost. Use of higher concentrations of capture and development reagents often increases sensitivity by one or twofold. Similarly, there is a linear relationship between how long an ELISA is allowed to develop and the sensitivity of that assay. For cytokines such as IL-5 or IL-13, produced at relatively high concentrations (typically 10-1000 pg/ml levels) following stimulation with strong recall antigens, the ELISA can sometimes be read at 1 h. For cytokines produced at levels where sensitivity is a major issue, such as IL-4, the assay is allowed to develop for up to 9 h. In all cases, the greater the difference between the absorbance of the maximum concentration of the recombinant standard and the background (all reagents except the cytokine), the better the precision of the assay.

Use of chemiluminescent detection systems rather than absorbance in ELISA results in additional 10 to 30-fold increases in sensitivity. The caveat is that an additional plate reader capable of quantitating chemiluminescence is required and the detection reagents for such assays are approximately 10-fold more expensive.

2. Materials

2.1. Buffers (see Note 1)

1. Coating buffer: 1.59 g Na_2CO_3 , 2.93 g NaHCO_3 made up to 1 L with double distilled water. Add NaN_3 to 0.02% and adjust pH to 9.6.
2. Blocking buffer: PBS containing 0.17% BSA, 0.02% NaN_3 . Adjust pH to 7.4.
3. Wash buffer: PBS containing 0.05% Tween-20. Adjust pH to 7.4.
4. Dilution buffer: PBS containing 0.085% BSA, 0.05% Tween-20, 0.02% NaN_3 . Adjust pH to 7.4.
5. Substrate buffer: 122 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 117 mL diethanolamine.

MgCl_2 should be dissolved in 800 mL double distilled water prior to addition of diethanolamine.

Diethanolamine may require warming at 37°C to liquefy. Adjust final pH to 9.8 and volume to 1 L. Keep this buffer in a dark bottle at 4°C as it is light sensitive. We utilize one *p*-nitrophenyl phosphate (PNPP) tablet (Sigma #S0942-200TAB) per 5 mL buffer.

The antibodies listed below are available from multiple sources including BD Pharmingen (<http://www.bdbiosciences.com>), Biolegend (<http://www.biolegend.com>), eBioscience (<http://www.ebioscience.com>), R&D Systems

(<http://www.rndsystems.com>), and Serotec (<http://www.serotec.co.uk>). Prices vary considerably for the same products. In several instances, the clones producing the Abs are available from the ATCC (<http://www.atcc.org>) providing a more cost-effective approach for labs which will make use of particular assays at high frequency. Here, we have listed combinations of clones, and conditions, that we find provide highly sensitive assays.

2.2. Measurement of Human IFN γ Levels

1. Capture antibody: mouse anti-human IFN γ monoclonal (clone MD-1).
2. Standard: human recombinant gamma interferon catalog number Gxg01-902-535. Available from NCI at 80,000 IU/ampoule.
3. Detection antibody: biotinylated mouse anti-human IFN γ monoclonal (clone 4S.B3).

2.3. Measurement of Human IL-2 Levels

1. Capture antibody: rat anti-human IL-2 monoclonal (clone MQ1-17H12).
2. Standard: interleukin-2, human, Jurkat derived 86/504 at 100 IU/ampoule available from NCI or NIBSC or recombinant IL-2 human, ISRO 851 at 1000 IU/ampoule available from NCI.
3. Detection antibody: biotinylated mouse anti-human IL-2 monoclonal (clone B33-2).

2.4. Measurement of Human IL-4 Levels

1. Capture antibody: mouse anti-human IL-4 monoclonal (clone 8D4-8).
2. Standard: recombinant IL-4 WHO standard 88/656 available from NCI or NIBSC at 1000 U/ampoule.
3. Detection antibody: biotinylated rat anti-human IL-4 monoclonal (clone MP4-25D2).

2.5. Measurement of Human IL-5 Levels

1. Capture antibody: rat anti-mouse/human IL-5 monoclonal (clone JES1-39D10).
2. Standard: human recombinant IL-5 or recombinant IL-5 WHO standard 90/586 from NCI or NIBSC at 5000 U/ampoule.
3. Detection antibody: biotinylated rat anti-human IL-5 monoclonal (clone JES1-5A10).

2.6. Measurement of Human IL-6 Levels

1. Capture antibody: mouse anti-human IL-6 monoclonal (clone MQ2-13A5).
2. Standard: human recombinant IL-6 (i.e., Peprotech #200-06).
3. Detection antibody: biotinylated mouse anti-human IL-6 monoclonal (clone MQ2-39C3).

2.7. Measurement of Human IL-10 Levels

1. Capture antibody: rat anti-human IL-10 monoclonal clone JES3-19F1 (HB 10487, from American Type Culture Collection or purchased from commercial source).

2. Standard: interleukin-10 WHO Reference Reagent 93/722 available from NIBSC at 5000 U/ampoule or human recombinant IL-10 from commercial source.
3. Detection antibody: clone JES-12G8 (HB 11676 from the American Type Culture Collection) or purchased.

2.8. Measurement of Human IL-11 Levels

1. Capture antibody: mouse anti-human IL-11 monoclonal (clone 22616.1).
2. Standard: human recombinant IL-11.
3. Detection antibody: biotinylated mouse anti-human IL-11 polyclonal (i.e., R&D Systems #BAF218).

2.9. Measurement of Human IL-13 Levels

1. Capture antibody: rat anti-human IL-13 monoclonal (clone JES10-5A2).
2. Standard: human recombinant IL-13 (Peprotech 200-13) or recombinant human IL-13 DNA WHO Reference Reagent 94/622 available from NIBSC at 1000 U/ampoule.
3. Detection antibody: biotinylated mouse anti-human IL-13 monoclonal (clone B69-2).

2.10. Measurement of Human TGF β Levels

1. Capture antibody: rat anti-mouse/human/pig TGF β 1 monoclonal (clone A75-2.1).
2. Standard: recombinant human TGF β 1 (Peprotech 100-21R) or transforming growth factor β -1, human, rDNA standard 89/514 at 3000 U/ampoule from NCI or NIBSC.
3. Detection antibody: biotinylated rat anti-mouse/human/pig TGF β 1 monoclonal (clone A75-3.1).

2.11. Measurement of Human TNF α Levels

1. Capture antibody: mouse anti-human TNF α monoclonal (i.e., Biolegend clone MAb-1).
2. Standard: human recombinant TNF α (Peprotech #300-01A).
3. Detection antibody: biotinylated mouse anti-human TNF α monoclonal (clone MAb11).

3. Methods

3.1. Assessment of Intra-assay Variability Because of Pipeting Error

Pipeting errors are cumulative, compounding the reduction in assay sensitivity by increasing variability in the assay. For this reason, the coefficient of variation (CV) of pipeting should be $\leq 3\%$ at each step. To assess this, ELISA solution from previous assays is used as a source of chromogen. For PNPP, a substrate for alkaline phosphatase, this yields a bright yellow solution. It is used to generate readings in the microplate plate reader, allowing one to determine how precisely and reproducibly one measures volumes.

For a diagram of suggested plate design and sample data, *see* **Table 1**.

Table 1
Determination of Coefficient of Variation: Sample Data

Row	Absorbance (405–690 nm)						
	1	2	3	4	5	6	7
A	0.899	0.919	1.779	1.764	3.887	3.866	3.869
B	0.934	0.887	1.766	1.779	3.28	3.251	3.276
C	0.912		1.757		1.66	1.682	1.697
D	0.927		1.762		0.869	0.871	0.861
E	0.907		1.771		0.437	0.429	0.43
F	0.893		1.77		0.229	0.225	0.223
G	0.905		1.747		0.121	0.122	0.115
H	0.919		1.802		0.067	0.067	0.061
Mean	0.912	0.903	1.769	1.772			
absorbance							
CV (%)	1.5	2.5	0.9	0.6			

1. Using P200 or similar equipment, pipet eight 50- μ L and eight 100- μ L volumes of expired ELISA solution into 96-well ELISA plates, using columns 1 and 3, respectively (running up and down plate).
2. In column 2, pipet two 50- μ L volumes of expired ELISA solution in the top two wells.
3. In column 4, pipet two 100- μ L volumes of expired ELISA solution in the top two wells.
4. In columns 5–7, add 50 μ L of dilution buffer to all 24 wells (three columns of eight wells each), then pipet 50 μ L of expired ELISA solution into the top three wells. Carry out twofold dilutions of this solution down the plate. Discard the 50- μ L extra from the last well.
5. Using microplate reader, determine the absorbance values at 405 nm (or more commonly 405–690 nm background) for each data set.
6. Using plate reader software (or by transferring data to SPSS, Excel, or similar software), determine the “CV” – the variability in dispensing and titrating samples – as a check on pipeting technique. Because errors are cumulative and ELISA is a multistep process, errors in dispensing samples and reagents need to be below 3% for maximal sensitivity.

3.2. Ultrasensitive ELISA

Having established that operator technique will add minimal variability to the assay, the optimized assay conditions below will provide excellent sensitivity (*see Note 2*). The specific capture and development reagents, standards, and their suggested concentrations are described below (*see Note 3*):

1. ELISA plate should be coated with 50 μL capture antibody in a high pH coating buffer at a predetermined optimal concentration, generally between 50 ng/mL and 2 $\mu\text{g/mL}$ (see **Note 4**). For maximum sensitivity, this incubation is carried out overnight at 40°C in tightly wrapped foil.
2. Plates with a sealing lid should be kept in a plastic box lined with wet paper towels to minimize evaporation, hence variability in edge wells (see **Note 5**).
3. Blocking, using 75 $\mu\text{L/well}$, should be carried out at 37°C for 1–3 h in a moist box (see **Note 6**). We find no difference in background between these times. This time period is used to prepare samples for the next step. Wash four times manually or using a plate washer. Afterwards, bang plate dry three times on paper towels to avoid random dilution of the next series of reagents added to the plate.
4. A standard curve of cytokine standard in dilution buffer is generated on each assay plate (see **Notes 7 and 8**). The titration range is determined by the anticipated intensity of your experimental values.
5. Each experimental sample should be titrated in dilution buffer from an empirically determined optimal starting dilution (typically between 1:2 and 1:10) for a series of four twofold 50- μL dilutions running down the plate (i.e., 1:2, 1:4, 1:8, 1:16) (see **Notes 9 and 10**). This allows ~20 sample titrations per plate. The initial dilutions should not sit in the starting well for more than 2 or 3 min before carrying out this titration, usually with a multichannel pipettor, or the resulting curves will not be linear. A sample of dilution buffer alone is generally included in wells A2, A3, and A4.
6. Sample incubation can be 3 h at 37°C or overnight at 40°C. Maximal sensitivity is obtained following overnight incubation. Wash 4 $\times$ as previously. Bang dry on paper towels.
7. Biotinylated development antibody in dilution buffer is added at a predetermined optimal concentration, generally between 50 ng/mL and 2 $\mu\text{g/mL}$, using 50 $\mu\text{L/well}$ (see **Note 11**). Incubation can be 3 h at 37°C or overnight at 40°C. Maximal sensitivity is obtained following overnight incubation. Wash 4 $\times$, banging dry 3 $\times$ on paper towels afterwards.
8. Streptavidin–enzyme conjugate is added at a predetermined optimal concentration to maximize signal while minimizing background. In our experience, streptavidin–alkaline phosphatase used at 1:1000 to 1:5000 with 50 $\mu\text{L/well}$ is useful. This incubation (45 min at 37°C) is followed by washing (4 $\times$), banging dry (3 $\times$), and addition of 50 $\mu\text{L/well}$ PNPP in substrate buffer at room temperature.
9. Progress of the assay is monitored visually, with readings (405–690 nm) taken at periods between 45 min and 6 h of assay development, depending on the sensitivity required.
10. A standard curve is prepared using the data obtained by serial dilution of the standard, with the cytokine concentration on the x axis (log) and the absorbance on the y axis (linear) to generate a sigmoidal curve.

When carrying out sample data analysis, the mean of at least two points (generally three or four points) *falling on the linear component of the standard curve* of that plate is required. These values should not differ from one another by more than ~15–20%, typically much less (see **Note 12**). Mean values $\pm$ standard

error for that sample should be reported. Interassay variability (the same samples titrated in an independent assay on another day) should be explicitly determined for a proportion of the samples to confirm reliability of the data/operator/assay. Sensitivity is defined in each assay as the beginning of the linear portion of the titration curve (*see Note 8*).

Unless statistical normality is demonstrated, a condition that usually requires large numbers of human subjects, the resulting population data should be analyzed by nonparametric statistics.

3.3. Optimized Conditions for Analysis of Human Cytokine Levels

3.3.1. IFN γ

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, mouse anti-human IFN γ , at 0.5 $\mu\text{g/mL}$.
2. For the standard, use human recombinant gamma interferon and titrate from 20 to 0.15 U/mL.
3. Dilute antigen-driven samples from 1:4. Titrate polyclonally stimulated cultures from 1:10.
4. Use the detecting antibody, biotinylated mouse anti-human IFN γ monoclonal, at 0.05 $\mu\text{g/mL}$.
5. Read the plate after 4 h with substrate for useful sensitivity of 0.15–0.3 U/mL.

3.3.2. IL-2

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, rat anti-human IL-2, at 2 $\mu\text{g/mL}$.
2. Use standard IL-2 and titrate from 10 to 0.078 U/mL.
3. Antigen-driven samples are diluted 1:2, whereas polyclonally stimulated cultures are diluted 1:10.
4. Use the detection antibody, biotinylated mouse anti-human IL-2, at 1 $\mu\text{g/mL}$.
5. Read plate after 5–6 h with substrate for sensitivity of 0.078–0.15 U/mL.

3.3.3. IL-4

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use capture antibody mouse anti-human IL-4 monoclonal at 0.4 $\mu\text{g/mL}$.
2. Use standard recombinant IL-4 titrated from 125 to 0.98 pg/mL.
3. Use antigen-driven samples either neat or at 1:2 dilution and polyclonally stimulated samples at 1:3 dilution.
4. Use detection antibody, biotinylated rat anti-human IL-4 monoclonal, at 0.06 $\mu\text{g/mL}$.
5. Read plate after 4–6 h with substrate to obtain sensitivity of 0.98 pg/mL or ~ 0.006 U/mL of WHO standard 88/656.

3.3.4. IL-5

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use capture antibody, rat anti-mouse/human IL-5, at 0.5 $\mu\text{g/mL}$.

2. Use standard human recombinant IL-5 between 1000 and 7.8 pg/mL.
3. Use antigen and polyclonally driven samples from 1:3 dilution.
4. Use detecting antibody, biotinylated rat anti-human IL-5, at 0.04 µg/mL.
5. Read plate after 2 h with substrate for sensitivity of 7.8 pg/mL or ~0.025 U/mL.

3.3.5. IL-6

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, mouse anti-human IL-6, at 2.5 µg/mL.
2. Use standard IL-6 and titrate from 2000 to 15.6 pg/mL.
3. Antigen-driven samples are diluted from 1:4, whereas polyclonally stimulated cultures are diluted from 1:10.
4. Use the detection antibody, biotinylated mouse anti-human IL-6, at 0.175 µg/mL.
5. Read plate after 2–3 h with substrate for sensitivity of 62.5 pg/mL.

3.3.6. IL-10

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use capture antibody, rat anti-human IL-10, at 2.5 µg/mL.
2. Use standard human recombinant IL-10 from 1000 to 7.8 pg/mL.
3. Use antigen-driven samples from 1:4 dilution and polyclonally stimulated cultures at 1:10 dilution.
4. Use detecting antibody at 1 µg/mL.
5. Read plate after 2 h with substrate for sensitivity of 7.8 pg/mL or ~0.01 WHO Reference Reagent 93/722 units.

3.3.7. IL-11

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, mouse anti-human IL-11, at 1.5 µg/mL.
2. Use standard IL-11 and titrate from 500 to 3.9 pg/mL.
3. Antigen-driven samples are diluted from 1:2.
4. Use the detection antibody, biotinylated mouse anti-human IL-11, at 0.08 µg/mL.
5. Read plate after 3–4 h with substrate for sensitivity of 7.8 pg/mL.

3.3.8. IL-13

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use capture antibody, rat anti-human IL-13, at 0.4 µg/mL.
2. Use standard human recombinant IL-13 from 1000 to 15.6 pg/mL.
3. Use antigen-driven samples at a dilution of 1:4 whereas use polyclonally stimulated cultures at 1:10 dilution.
4. Use detecting antibody, biotinylated mouse anti-human IL-13, at 0.2 µg/mL.
5. Read plate after 4–6 h with substrate for sensitivity of ~15 pg/mL.

3.3.9. IL-13 Ultra High Sensitivity Assay

Typical food antigen-driven responses (i.e., peanut-specific) fall between 10 and 200 pg/mL and altered ELISA conditions may be used to yield an assay with higher sensitivity.

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use capture antibody, rat anti-human IL-13, at 1 $\mu\text{g/mL}$.
2. Use standard recombinant IL-13 from 200 to 1.56 pg/mL .
3. Use detecting antibody, mouse anti-human IL-13, at 0.2 $\mu\text{g/mL}$.
4. Read plate after 6 h with substrate for sensitivity of 0.78 pg/mL .

3.3.10. *TGF β*

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use capture antibody, rat anti-mouse/human/pig TGF β 1, at 1 $\mu\text{g/mL}$.
2. Use standard recombinant human TGF β 1 from 2000 to 15.6 pg/mL .
3. Prior to assay, serum/culture samples are acidified to convert TGF β 1 from latent to active form for recognition in this assay. Incubate 50 μL of sample with 2 μL 1 N HCl for 15 min at room temperature; then add 2 μL 1 N NaOH. Carry out dilutions from 1:5 (which should be later calculated based on an actual dilution of 1:5.4, allowing for the addition of acid and base above).
4. Use detecting antibody, biotinylated rat anti-mouse/human/pig TGF β 1, at 250 ng/mL .
5. Read plate after 5 h with substrate for sensitivity of $\sim$ rDNA TGF β 1 standard 89/514 U/mL.

3.3.11. *TNF α*

1. Using the protocol for ultrasensitive ELISA (*see Section 3.2.*), use the capture antibody, mouse anti-human TNF α , at 1.0 $\mu\text{g/mL}$.
2. Use standard TNF α and titrate from 2000 to 15.6 pg/mL .
3. Antigen-driven samples are diluted from 1:4, whereas polyclonally stimulated cultures are diluted from 1:10.
4. Use the detection antibody, biotinylated mouse anti-human TNF α , at 0.5 $\mu\text{g/mL}$.
5. Read plate after 3 h with substrate for sensitivity of 60 pg/mL .

4. Notes

1. All solutions are kept in a cold room and are stable for a minimum of 6 mo.
2. In initially developing an ELISA, it needs to be remembered that arbitrarily pairing two cytokine-specific monoclonal antibodies or a monoclonal and polyclonal antibody will not necessarily yield a useful assay. This is a largely empirical process that may require testing of several different antibody combinations to obtain an assay with high sensitivity.
3. Routinely, most ELISA reagents are reconstituted in saline as recommended by the manufacturer, then diluted with an equal volume of high-quality glycerol and stored at -20°C where they are stable for at least 1 yr. Addition of glycerol allows the Ag, Ab, and enzyme conjugates to be stored at this temperature and used without undergoing multiple freeze thaw cycles.
4. A variety of high-binding EIA plates are commercially available. We have found substantial differences in their ability to bind capture antibody or Ag. Costar 3369 plates work effectively in our assays.
5. "Edge" effects, most commonly values that are too high, result from evaporation of edge wells. The solution is to ensure plates are kept tightly wrapped in a humidified

chamber (plastic box with wet paper towels) for all incubations. Also, if samples are allowed to sit in plate for an extended period prior to carrying out the dilutions, assay reproducibility is reduced.

6. A broad variety of blocking reagents (skimmed milk, bovine serum albumin (BSA) or gelatin) can be used. Recommended wash buffers sometimes utilize very high concentrations of BSA and Tween-20. In our experience, BSA at 0.17% (1.7 g/L) in PBS with 0.05% Tween-20 is sufficient to minimize backgrounds with less cost and pollution of the environment than buffers containing up to 5% BSA and 2% Tween-20.
7. Biological standards are calibrated in units of biological activity which are established by consensus following exhaustive collaborative studies involving many laboratories. They have the advantage of allowing ready comparison of cytokine levels between different laboratories using a common standard. There is a hierarchy of standards, with the WHO International Standard being the primary standard. These standards are calibrated in arbitrary units, usually based on an initial functional bioassay, to provide a common standard that can be used to compare data internationally. It is vital for international harmonization that secondary standards are calibrated against these primary standards. Secondary standards may be national or regional standards or working reference materials used in the laboratories of manufacturers, regulators, or others. WHO reference standards are available for most cytokines. There are only 4 "official" custodian laboratories for WHO International Standards and these are NIBSC, CLB in the Netherlands, CDC, and NIH. NIBSC is a multidisciplinary scientific establishment located in the UK which has a national and international role in the standardization and control of biological substances used in medicine. As a WHO International Laboratory for biological standards it prepares, evaluates, and distributes ampouled biological reference substances which serve the pharmaceutical, regulatory, and research communities as International Standards. Similarly, the BRB Preclinical Repository is a (US) NCI-sponsored facility which contains bulk cytokines, monoclonal antibodies, and cytokine standards. Its purpose is to maintain a constant and uniform supply of high-quality reagents for scientists at nonprofit as well as commercial establishments. Information on free distribution (other than a nominal shipping charge) of these standards is available from the NIBSC at <http://www.nibsc.ac.uk/catalog/standards> or the US NCI at <http://www.ncicrf.gov/brb/preclin>.

Many laboratories choose to utilize recombinant standards from well-known manufacturers (i.e., BD Pharmingen, Peprotech, R&D Systems) and instead express their data in mass units based on that commercial preparation and lot. Because such standards vary widely in specific activity, some manufacturers have calibrated the specific activity of their standards against the WHO standard. Others utilize the bioassay of their choice to obtain specific activities. It should be recognized that 10^6 U/mL in one assay is not necessarily equivalent to 10^6 U/mL in a different assay. Comparison between, for instance, two IL-4 preparations from different sources can easily be 100-fold different in terms of specific activity. In practice, many investigators use polyclonal activators to generate 30–100 mL of bulk tissue

culture supernatant containing high concentrations of natural cytokine that can then be calibrated against WHO standards for routine usage as a laboratory standard. If this approach is selected, one should be aware that different polyclonal activators are optimal for inducing intense Th1 (i.e., soluble anti-CD3 at 30 ng/mL, Pharmingen; TSST-1 at 10 ng/mL, Sigma), Th2 (i.e., PHA at 10 µg/mL, Sigma), or monokine (SAC, formalin-fixed *Staphylococcus aureus* from Sigma at 0.01–0.1 µg/mL) associated cytokine production.

8. When assay sensitivity is not sufficient for a given application, the first step is to compare the sensitivity of the standard curve generated with those found in the literature or detailed above. Assuming you obtain similar sensitivity to others, you need an estimate of the cytokine levels expected. Some cytokines are produced at substantial levels (i.e., IL-5, IL-13) whereas others, notably IL-4, are very tightly regulated and present at pg/mL levels in antigen-stimulated cultures.
9. If experimental values do not agree throughout the titration range, it is possible that the dilution range selected is not within the linear range of the assay. The titration of the samples needs to be adjusted so that more of the points lie on the linear component of the standard curve.
10. When a trainee is first gaining experience with ELISA, we provide coded samples of known concentration that fall within the range of values typically obtained in experimental work. These are titrated in duplicate until the accurate concentration ($\pm 10\text{--}15\%$) is reproducibly determined.
11. Development antibodies directly conjugated with enzyme are rarely used in ultra-sensitive ELISA as they do not provide adequate signal. Biotinylated reagents yield at least a 10-fold increase in sensitivity. Avidin/streptavidin enzyme conjugates generally utilize alkaline phosphatase or horse radish peroxidase. The methods presented above use alkaline phosphatase with PNPP substrate as it is inexpensive, stable, and generates a robust signal that increases linearly over many hours.
12. The most common reason for duplicates which do not agree is poor titration precision/operator error.