Video Article

Determination of the Relative Potency of an Anti-TNF Monoclonal Antibody (mAb) by Neutralizing TNF Using an *In Vitro* Bioanalytical Method

Lilia Tierrablanca-Sánchez[1], Víctor Pérez Medina Martínez[1], Nancy D. Ramírez Ibañez[1], Néstor O. Pérez Ramírez[1], Francisco Luis Flores Ortiz[1], Emilio Medina-Rivero[2]

[1]Research and Development, Probiomed

[2]Bioprocess Research and Development Unit, Instituto Politecnico Nacional

Correspondence to: Francisco Luis Flores Ortiz at luis.flores@probiomed.com.mx, Emilio Medina-Rivero at emrivero@yahoo.com.mx

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Abstract

This protocol shows the measurement of the apoptotic activity neutralization of TNFα in a mouse fibroblast cell model (WEHI 164) using an anti-TNFα mAb. In addition, this protocol can be used to evaluate other anti-TNFα molecules, such as fusion proteins. The cellular model employed here is sensitive to TNFα-mediated apoptosis when an additional stress factor is induced in cell culture conditions (*e.g.,* serum deprivation). This procedure exemplifies how to execute this analytical assay, highlighting the key operations relating to the sample preparation, cell dilution, apoptosis induction, and spectrophotometric measurements that are critical to ensure successful results. This protocol reveals the best-performance conditions relating to apoptosis induction and efficient signal recording, leading to low uncertainty values.

Video Link

The video component of this article can be found at https://www.jove.com/video/55376/

Introduction

Biological potency is the quantitative measure of biological activity based on the assayed product attributes that are linked to the relevant biological properties, whereas quantity (expressed in mass) is a physicochemical measure of protein content. Potency tests, along with other analytical methodologies, are performed as part of product conformance, stability, and comparability studies. In this sense, potency measurements are used to demonstrate that product batches meet the critical quality attributes (CQAs) or acceptance criteria during all phases of clinical trials and after market approval.

Apoptosis is programmed cell death, naturally occurring when cells are infected with a virus or when the cells are stressed by an environmental factor that compromises cellular viability and function[1,2]. Among others, apoptosis inhibition, or biological neutralization, is one of the principally known therapeutic mechanisms of mAbs, particularly in the treatment of chronic diseases, such as immune-mediated inflammatory disorders. Anti-TNFα molecules exert their therapeutic properties by blocking the interaction of tumor necrosis factor alpha (TNFα) with the p55 and p75 cell surface receptors[3], thus preventing signal pathways that finally lead to cellular apoptosis.

TNFα can produce inflammation in some chronic illnesses[4]. TNFα is spuriously secreted into the extracellular milieu by macrophages, which are sentries of the innate immune system and the main actors in this kind of disease[5]. As a common path, TNFα deregulation is associated with the pathogenesis of these illnesses.Without control and under constant induction and cell stress, TNFα induces cell death and tissue degeneration, ultimately leading to rheumatoid arthritis, Crohn's disease, and other pathological profiles[6].

TNF antagonists that block the interaction between TNF and its receptors have been increasingly used as an effective therapy to reduce symptomatology and hinder the progression of these diseases. Nowadays, anti-TNFα drug products are widely used to control the systemic concentration of this cytokine, thus preventing further degeneration of involved tissues. In this sense, providing a reproducible and robust bioassay to describe the specific ability of a drug to achieve its biological effect is imperative.

In this protocol, critical steps-identified during the development of a neutralization assay-for the successful measurement of biological potency are highlighted, with a particular emphasis on the skills needed to execute the bio-analytical method. This bio-analytical method provides useful comparability information between different batches or anti-TNFα drug products when compared to a clinically tested reference substance.

Protocol

1. Preparation of the Media and Solutions

1. Prepare the culture medium: RPMI-1640 with 10% FBS, pH 7.4.
2. Prepare assay culture medium: RPMI-1640 without phenol red but with 1% FBS, pH 7.4.
3. Prepare cell wash solution: DPBS Mg- and Ca-free solution with 0.02% EDTA, pH 7.4.
4. **Prepare cell detachment solution: 0.125% trypsin with 1 mM EDTA.**
    1. Thaw 100 mL of a 0.25% solution of trypsin-EDTA and transfer to a sterile 500-mL flask.
    2. Mix with 100 mL of cell wash solution and dispense 15 mL aliquots into 15 mL sterile tubes. Store at -70 to -80 °C until use.
    3. Filter these solutions through a 0.22-µm membrane and warm up to 37 °C for at least 30 min prior to use.

5. **Prepare apoptosis-induction stock solution TNFα solution at 3.3 µg/mL.**
    1. Dissolve 20 µg of TNFα with 500 µL of filter-sterilized water in its primary container and mix until complete dissolution.
    2. Transfer into a 15 mL sterile tube and add 5.5 mL of DPBS Mg- and Ca-free solution to this tube. Mix gently using a vortex mixer.
    3. Aliquot the solution into 70 µL portions. Dispense each aliquot into 0.5 mL microtubes and store at -80 °C.

6. **Prepare apoptosis induction solution : TNFα solution at 40 ng/mL.**
    1. Thaw an aliquot of the apoptosis induction stock solution, incubating it in a water bath at 25 ˚C for 10 min.
    2. Dilute the apoptosis induction stock solution to 40 ng/mL by adding 61 µL of 3.3 µg/mL TNFα solution to 4.939 mL of assay culture medium in a 15 mL sterile tube.
    3. Mix by vortex mixer for 10 s; this solution must be prepared freshly before use.
    4. Warm up the solution to 37 °C for at least 30 min prior to use in th eneutralization assay.

7. **Prepare the substrate solution: caspase 3/7 Glo solution[7,8].**
    1. Thaw the caspase buffer solution (caspase 3/7 Glo buffer) 12 h before use.
    2. Let the caspase buffer solution and the substrate (caspase 3/7 Glo substrate) sit separately at 25 ± 5 °C for 30 min prior to mixing.
    3. Transfer 10 mL of the caspase buffer solution to the substrate vial and mix by inversion.
    4. Keep at 25 ± 5 °C, light-protected until use.
    NOTE: The solution is stable for 6 h at room temperature.

2. Cell Culturing and Counting

1. **Cell thawing and the first subculture.**
    1. Remove one vial with WEHI 164 cells[9] from a freezer at -80 ˚C and transfer them to an ice-bath.
    2. Pipette up and down with 1 mL of pre-warmed culture medium until the frozen cells completely thaw.
    3. Dispense 9 mL of pre-warmed culture medium onto a 15 mL sterile tube.
    4. Transfer the cell suspension into the 15-mL sterile tube and mix gently five times by inversion.
    5. Centrifuge the cell suspension at 125 x *g* for 3 min. Discard the supernatant and disaggregate the cell pellet.
    6. Add 5 mL of culture medium to the tube . Mix until the cells are completely resuspended.
    7. For cell counting, transfer 50 µL of the cell suspension to a 500 µL microtube and mix with 50 µL of 0.4% trypan blue. Count the cells and adjust to 0.5 x 10^6 cells/mL. See step 2.2, below.
    8. Add 13 mL of pre-warmed culture medium to a 75 mL cell culture flask.
    9. Dispense enough cell suspension volume from step 2.1.6 to achieve 0.5 x 10^6 cells/mL in the cell culture flask and incubate at 37 °C and 5% CO_2 overnight.

2. **Cell counting.**
NOTE: See reference[10].
    1. Using the solution from step 2.1.6, transfer 0.05 mL to a hemocytometer and determine the cell density under a microscope using trypan blue exclusion.
    2. Quantify the total number of cells and viable cells.
    3. Adjust the cell suspensions to 0.5 x 10^6 cells/mL.
    Equation 1
    $$V_{\text{culture medium}}(\text{mL}) = \left(\frac{\frac{0.5 \times 10^6 \text{cells}}{\text{mL}} * 5 \text{ mL}}{\text{NVC cells/mL}} \right)$$
    $V_{\text{culture medium}}$(mL) = (5mL - $V_{\text{cell suspension}}$)
    $V_{\text{culture medium}}$(mL) = Adjusted volume of WEHI 164 cell suspension
    NVC = Number of viable WEHI 164 cells/mL
    $V_{\text{culture medium}}$(mL) = Assay culture medium volume added to the cell suspension to achieve 0.5 x 10^6 cells/mL
    0.5 x 10^6 = Target cell density

3. **Cell detachment and the second and third subculture.**
NOTE: A vacuum system can be used to remove the solutions from the flasks. Disposable or glass sterile pipettes can be used. If the pipette has a cotton clog at the top, it must be removed before use.
    1. Remove the culture medium from the cell culture T-flask using a 1 mL sterile pipette and a vacuum.
    2. Dispense 5 mL of cell wash solution into the culture T-flask, mix gently, and discard the solution. Repeat this step twice.
    NOTE: The complete removal of the culture medium is critical for efficient cell detachment.
    3. Add 15 mL of cell detachment solution to the T-flask and let stand for 3 min in an incubator at 37 °C and 5% CO_2.

4. Verify the absence of attached cells in the flask inner wall under the microscope. Remove the cells from the culture T-flask using a 20 mL sterile pipette and dispense them into a 50-mL sterile tube.
5. Centrifuge the cell suspension at 125 x *g* for 3 min. Discard the supernatant and resuspend the pellet with another 5 mL of culture medium.
6. Count the cells and add enough culture medium to reach the desired cell concentration according to **Equation 1**.
7. Add this suspension to a 72 cm^2 T-flask and incubate overnight at 37 °C and 5% CO_2.
8. Subculture the cells at least two times before using them in the neutralization assay. Repeat steps 2.3.1-2.3.8 for the next two days.

4. **Assay cell suspension.**
   1. Select a WEHI 164 subculture that has at least three passes. See step 2.1.
   2. Detach and count the cells according to steps 2.2 and 2.3 of this protocol.
   3. Dilute the cell suspension according to **Equation 1** to 0.5 x 10^6 cells/mL.
   4. Use this cellular suspension for the neutralization assay. Mix all cell suspensions by vortex mixer prior to use.

3. Antibody Preparation and Dilutions

1. **Quantitation of the mAbs.**
   1. Determine the concentration of reference substance, control sample, and analytical sample through UV absorption at 280 nm using their mass extinction coefficient (1.39)[11].
      NOTE: Original concentrations could be taken from drug product labels. However, this must be verified by UV absorption.

2. **mAb dilutions.**
   1. Dilute all samples independently in triplicate, with DPBS Mg- and Ca-free solution in 2 mL microtubes, down to 2 mg/mL. Confirm this concentration by UV absorption in triplicate, using DPBS Mg- and Ca-free solution as the blank.
   2. Mix the stock protein solutions for 5 s using a vortex mixer.
   3. Dilute 100 µL of each 2 mg/mL mAb solution with 0.9 mL of the assay culture medium.
   4. Mix for 5 s by vortex mixer.
      NOTE: These solutions have a concentration of 200 µg/mL. Dilutions must be done for each triplicate.
   5. Dilute 10 µL of each 200 µg/mL mAb solution with 0.99 mL of the assay culture medium. Mix for 5 s using a vortex mixer. These solutions have a concentration of 2 µg/mL. Perform serial dilutions for each triplicate before using them in the neutralization assay.
   6. Make anti-TNFα mAb dilutions in three independent microplates. Make a duplicate from each independent triplicate and dispense them in one microplate, as indicated in **Table 1**. Reference Substance

Plate 1		Plate 2		Plate 3	
Wells	Sample	Wells	Sample	Wells	Sample
B2:B11	Reference Substance	B2:B11	Control Sample	B2:B11	Analytical Sample
C2:C11		C2:C11		C2:C11	
D2:D11	Analytical Sample	D2:D11	Reference Substance	D2:D11	Control Sample
E2:E11		E2:E11		E2:E11	
F2:F11	Control Sample	F2:F11	Analytical Sample	F2:F11	Reference Substance
G2:G11		G2:G11		G2:G11	

Table 1: Microplate sample arrays. A complete neutralization assay must be run in three microplates within coordinates B2 to G11. Random dispensing of reference, analytical, and control samples allow researchers to verify any bias in the assay.

   7. Perform mAb dilutions of each reference, sample, or control, as shown in **Table 2**.
      NOTE: The anti-TNFα mAb concentrations described in this table are not the final concentrations in the neutralization assay.

Plate Column	Volume of Assay culture medium (µL)	Volume of Reference Substance, Analytical Sample or Control Sample (uL)	Concentration in the Assay Plate (ng/mL)
2	0	230	2000
3	150	150 from line 2	1000
4	75	75 from line 3	500
5	100	50 from line 3	333
6	75	75 from line 4	250
7	75	75 from line 5	166
8	75	75 from line 6	125
9	75	75 from line 7	83
10	75	75 from line 9	41
11	150	75 from line 10	13

Table 2: Anti-TNFα mAb dilutions. Serial dilutions of anti-TNFα mAbs are demonstrated in this table. Final concentrations described in this table are not the concentrations in the assay, where anti-TNFα mAbs were diluted by a factor of 3 (mAb dilution + culture medium + cells suspension). Lines in bold represent dilutions coming from lines 3, 5, 7, 9, and 10; non-bolded lines represent dilution from lines 3, 4, and 6. These serial dilutions are done just before performing the neutralization assay. Care must be taken to mix by pipetting up and down three times before dispensing the dilutions.

8. Keep the plates at 25 ± 5 °C until use.

4. Neutralization Assay with WEHI 164 Cells

1. Mix by vortexing all cell suspensions (0.5 x 10^6 cells/mL) prior to dispensing at any step of this protocol.
   NOTE: In this section, warm each solution to 37 °C for 30 min prior to use.
2. Transfer 50 µL of the cell suspension to each of the 60 wells of the microplates, moving from column 2 to 11 and line B to G.
3. Transfer 50 µL of mAb reference, sample, and control dilutions into microplates. Follow the pattern depicted in **Figure 1**.
4. Add 50 µL of the apoptosis induction solution to each well.
5. Use cellular controls of 50 µL of WEHI 164 cells, dispensed in three wells. Bring each well to a final volume of 150 µL with assay culture medium.
6. Use a cytotoxicity control of a mixture of 50 µL of WEHI 164 cells plus 50 µL of apoptosis induction solution. Bring each well to a final volume of 150 µL with assay culture medium.
7. For the TNFα control, use 50 µL of the apoptosis induction solution and bring it to 150 µL with the assay culture medium.
8. For the blank, use 150 µL of the assay culture medium alone.
9. Fill the remaining wells with 150 µL of culture medium to avoid plate evaporation effects.
10. Repeat steps 4.1.1-4.1.9 twice in two other microplates.
    NOTE: The mAb final concentrations in the microplate are:0.666, 0.333, 0.167, 0.111, 0.083, 0.056, 0.042, 0.028, 0.014, and 0.004 µg/mL.
11. Load the samples in microplates, as indicated in **Figure 1**.

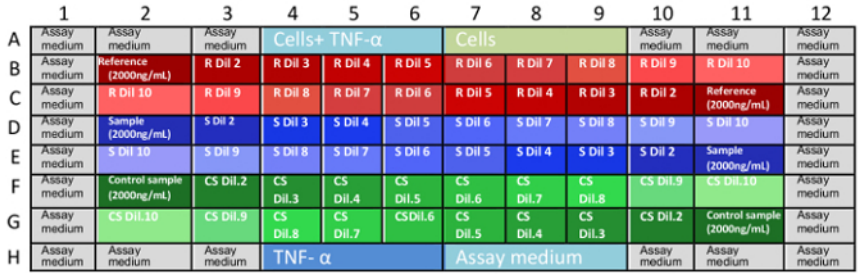


Figure 1: Disposition of samples in the assay plates. B1 to G11 are well coordinates in the microplates and describe the positions where the sample dilutions are placed. Missing coordinates are wells filled with controls and assay culture medium (A1-A12 and H1-H12). This random distribution of samples (forward and reverse dilutions in the microplates) helps to eliminate bias in the results due to the evaporation of medium or other variables. It is best that each microplate is done by one analyst at a time. R: Reference, S: sample, CS: control sample, Dil: dilution. Please click here to view a larger version of this figure.

12. Incubate the three plates at 37 °C and 5% CO_2 for 16 ± 2 h.
13. Let the caspase 3/7 Glo reagent stand at 25 ± 5 °C for 30 min before use.
14. Add 100 µL of this reagent to all wells, including the samples and controls.
15. Shake the plates using a microplate vortex mixer for 3 min at 25 ± 5 °C immediately after dispensing into the wells.
16. Incubate the plates for 2.5 ± 0.5 h at 25 ± 5 °C, protected from light.

17. Insert the microplates into the luminometer and complete the next section.

5. Analysis of Results

1. Using a software for luminescence detection, select the luminescence mode and endpoint function.
2. Select a 96-well clear-bottom plate and its 80 internal wells, excluding columns 1 and 12.
3. Select an integration time of 1,250 ms and 10 s for mixing the microplate before reading.
4. Select the wells where reference substance, analytical substance, and control sample will be placed and identify with their corresponding concentrations.
5. Read the samples placed in the microplates with the luminometer.
6. Use a fourth parameter equation for the analyses of results. Graph a dose-response curve, as depicted in **Figure 2**.

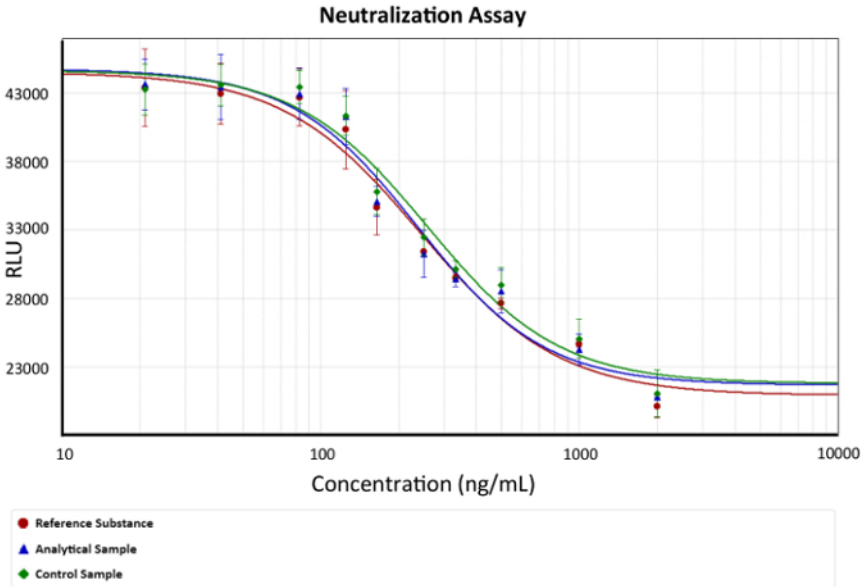
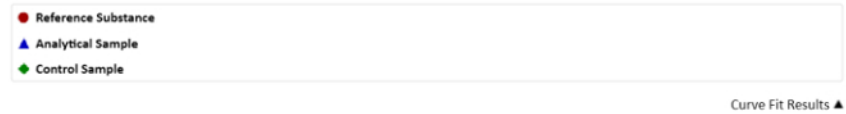


Figure 2: Dose-response curve. Anti-TNFα mAb concentration versus luminescence (cell viability) is depicted. A fourth parameter equation describing the anti-TNFα protection of mAbs was used as a model. EC50 is the concentration of mAb that can neutralize the amount of TNFα that cause 50% cell death in each assay, exemplified in the graph as the change in slope. Bars describe the standard deviation of luminescence for each mAb concentration. *x* represents anti-TNFα Ab concentration and is depicted as a logarithmic function in ng/mL, while *y* represents the luminescence response in arbitrary luminescence units. Please click here to view a larger version of this figure.
NOTE: In the fourth parameter equation, C is the effective concentration 50 (EC50). This value will be used to compare the reference substance, analytical sample, and control sample by means of the effector function.

7. For calculating the relative potencies, fix the reference substance to 100% and calculate the potencies of the sample and control accordingly.
NOTE: Those values are depicted in **Figure 3**.



Curve Fit Results ▲

Curve Fit : 4-Parameter $y = D + \frac{A-D}{1+\left(\frac{x}{C}\right)^B}$

	Parameter	Estimated Value	Std. Error	Confidence Interval
Reference Substance $R^2 = 0.973$ EC50 = 245.2	A	4.45e+4	1693	[4.04e+4, 4.87e+4]
	B	1.634	0.460	[0.507, 2.760]
	C	245.2	40.46	[146.2, 344.2]
	D	2.09e+4	2041	[1.59e+4, 2.59e+4]
Analytical Sample $R^2 = 0.971$ EC50 = 238.2	A	4.47e+4	1670	[4.07e+4, 4.88e+4]
	B	1.769	0.507	[0.528, 3.009]
	C	238.2	38.25	[144.6, 331.8]
	D	2.17e+4	1934	[1.69e+4, 2.64e+4]
Control Sample $R^2 = 0.972$ EC50 = 259.9	A	4.47e+4	1592	[4.08e+4, 4.86e+4]
	B	1.712	0.490	[0.513, 2.911]
	C	259.9	42.65	[155.6, 364.3]
	D	2.18e+4	2020	[1.69e+4, 2.67e+4]

Figure 3: Mathematical equation used for calculating the EC50s and their values. EC50 values, or C parameters, have their uncertainty depicted as standard error. A comparison of EC50s between the sample and reference results of relative potency is also depicted. The confidence interval is calculated with an α = 0.05. Please click here to view a larger version of this figure.

Representative Results

Dose-response Graph (with Controls)

Figure 1 represents the luminescence response versus mAb concentration. This sigmoidal function exemplifies caspase 3 and 7 release in the assay culture medium due to cell lysis. Cell death is enhanced by serum starvation plus TNFα signaling induction. Therefore, the anti-TNFα molecule (mAb) interacts with the cytokine, inhibiting (by steric hindrance) its interaction with the TNF cell receptor. This causes cell survival at higher mAb concentrations.

The controls used in the method were: cells with assay culture medium, cells plus TNFα plus assay culture medium, and assay culture mediumalone. Cells alone with assay culture medium under FBS starvation did not undergo to apoptosis, thus developing luminescence. Moreover, cells exposed to TNFα alone but cultivated with the culture medium did indeed survive.

Another important control is the assay culture medium alone. This controls helps with the understanding of molecular interference relating to the medium, specifically proteins that can digest the caspase substrate, thus indicating a false-positive luminescent signal. The EC50 and relative potency results are depicted in **Table 3**.

Sample	EC50	Relative Potency (%)	Confidence Interval (%)	RDS (%)
Reference substance	241.5	100	--	--
	243.6			
	234.2			
Analytical Sample	225.2	99.7	86.0-115.1	8.5
	240.3			
	258.8			
Control Sample	230.5	97.1	86.5-108.7	6.9
	264			
	248.4			

Table 3: Relative potency results. The analytical sample is compared to a known potency sample, described in the table as the reference, with a fixed 100% potency. The relation is calculated with the EC50 of each sample. The interval is calculated at a 95% confidence (α = 0.05). RSD: relative standard deviation.

Table 3 shows the relative potency, in percentage, between a reference mAb and a sample under investigation. Comparability is assumed within a range of 80-120% of the reference. However, sometimes the reference has large variability between batches; therefore, the range of acceptance can change. Hence, it is desired to select reference batches within a short period of manufacturing, tightening the physicochemical properties and acceptance interval.

This table shows that the reference has a potency of 100%, while the analytical sample has a potency of 99.7%. This result means that the TNFα neutralization capability by the sample is comparable to that of the reference. It is expected that illnesses related to the overexpression of the cytokine can be controlled by these mAbs.

Discussion

This characterization helps to determine *a priori* the biological behavior of a molecule under development before expensive and time-consuming clinical trials are conducted. It is also useful for the batch-to-batch release of an approved drug product. It is worth mentioning that these assays are useful for determining if a molecule has an adequate biological effect regarding its mechanism of action. The bio-analytical method presented in this tutorial is critically important to the comparison of different anti-TNFα molecules. Despite the common physicochemical method, this methodology is able to determine, through biological means, the potency and efficacy of a drug as a quality attribute, thus showing the complete and full significance of the effector functions.

Commonly, cell apoptosis responsiveness to TNFα can be a challenging task for researchers to perform. Troubleshooting can be conducted through the characterization of the cell bank that is used on a daily basis before standardizing this biological method. One example is the cell response variability within a time period relating to cell-line aging[12]. This problem is eliminated using a master cell bank frozen at -80 °C. The working cell bank must be large enough to cover the requirements of a DOE study performed by R&D or a one-year period for use in the quality-control laboratory. Also, this responsiveness can be fixed using temperature-stabilized solutions before adding them to cells at any step during this protocol. At least three passes must be performed before running a neutralization assay and restricting the length of time that cells are grown in continuous culture due to adaptation and population dynamics[12,13].

TNFα molecule must be protected from freeze-thaw cycles, as the potency of this protein is substantially undermined if not stabilized. Formulations cited elsewhere[14] for cytokine preparation are suggested when the reconstituted cytokine will be stored, as the concentration of TNFα is critical for protocol success. Responsiveness of a cell to a cytokine depends on the number of receptors per cell. Therefore, the optimal

TNFα concentration depends on the cell line and density[15,16]. We diluted TNFα to a final concentration of 13.3 ng/mL and adjusted the cell density using a curve around 25,000 cells/well. Therefore, the cell density must be verified for each TNFα concentration.

Camacho-Villegas *et al.* reports a TNFα final concentration of 1.25 ng/mL; this group also uses actinomycin D as a stress cell factor[9,17,18]. We instead changed the FBS from 10% to 1% from culture medium to assay medium, giving a strong stress signal to induce apoptosis in WEHI 164 cells with TNFα alone, eliminating another variable from the protocol. Other groups report cell viability using MTT. Methodologies using a luminescence substrate sensitive to caspase 3[7], instead of the spectrophotometric method where the UV-Vis absorbance substances are present in the culture medium or the cells themselves (*e.g.,* phenol red absorbed by cells), can interfere with the signal. Thus, the sensitivity of the assay was increased using this Ac-DEVD-pNA luminescentreactant, as the presence of luminescent substances (background) in the culture medium is not expected.

A limitation of this method is that it can only be applied to TNFα-sensitive cells; other cells lines must be tested and the cytokine concentration adjusted for an optimized cellular response. Furthermore, the absolute response cannot be measured, as we are not using a primary cell line or an *in vivo* assay; instead, orthogonal isothermal titration calorimetry (ITC) experiments are suggested for initial method adjustment. Information from ITC is helpful for establishing TNFα affinity with a new molecule under development and for specifying the initial conditions in the biological method.

This method is useful for testing new molecules when researchers have a reference substance for obtaining a basal relative response; thus, the evaluation of a bio-better or a molecular response relating to cell protection is recommended. It can be applied to other anti-TNFα proteins. For instance, it is applicable to evaluating the relative biological potency of Etanercept, Infliximab, Certolizumab, or Golimumab[19]. However, all these anti-TNFα molecules have different affinities for the cytokine; therefore, their concentrations must be adjusted individually. Another advantage of this method is the short time between the initiation of experiments and the achievement of results, making it easy to execute and inexpensive compared to animal models. Further, this method measures interaction of the mAb with a fully active TNFα molecule, suggesting that the mAb is recognizing the TNFα trimer and that the molecules were not modified during storage or laboratory manipulation. On the other hand, a pure physicochemical assay result is still dubious. For example, interaction between TNFα and a mAb using a conventional ELISA can be an artifact relating to structural changes due to cytokine chemical immobilization; therefore, the affinity could be affected and the measurements compromised. Moreover, during ITC analyses, dilution solutions can modify the structure of TNFα or the mAb, thus inhibiting TNFα trimer formation, mAb interaction, or artifactual epitope generation. The use of primary cells lines in this method could be demanding; nonetheless, protection against TNFα can give interesting results, mimicking *in vivo* responses.

We designed this method to be easy to follow and reproducible. As luminescence cannot be masked by phenol red or other UV-Vis absorbance substances, almost every culture medium additive can be used for cultivating cells. This modification aids researchers in the study of demanding cell lines, with a full detection response for viability after cytokine treatment. At least three cell passages and cell density adjustments must be conducted before executing the neutralization assay. Also, stabilizing the cytokine and its concentration, as well as warming and equilibrating the CO_2 concentration in the culture medium and solutions are critical steps for assay success. Overall, this article shows the steps necessary to neutralize TNFα cytokine with a mAb using an *in vitro* biological test to compare a reference and a sample under development.

Disclosures

The authors have nothing to disclose

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