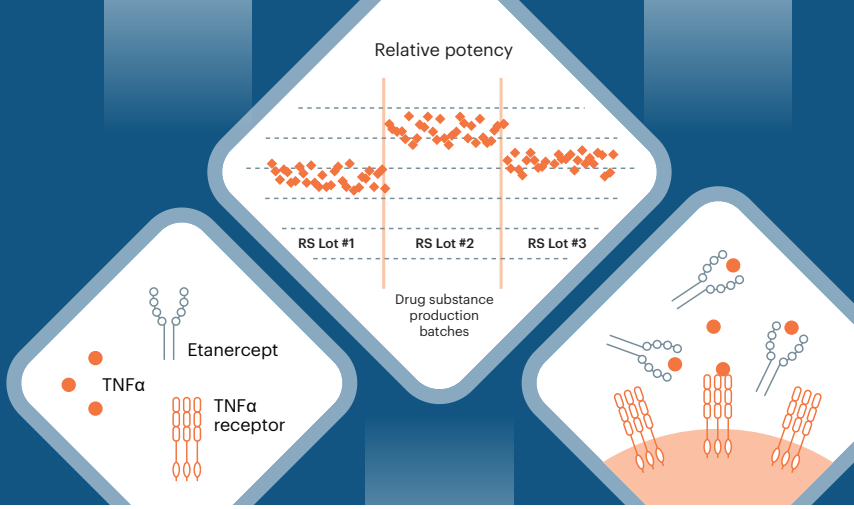
Application Note

Etanercept for bioassays reference standard: the key to consistent and reliable potency assessment



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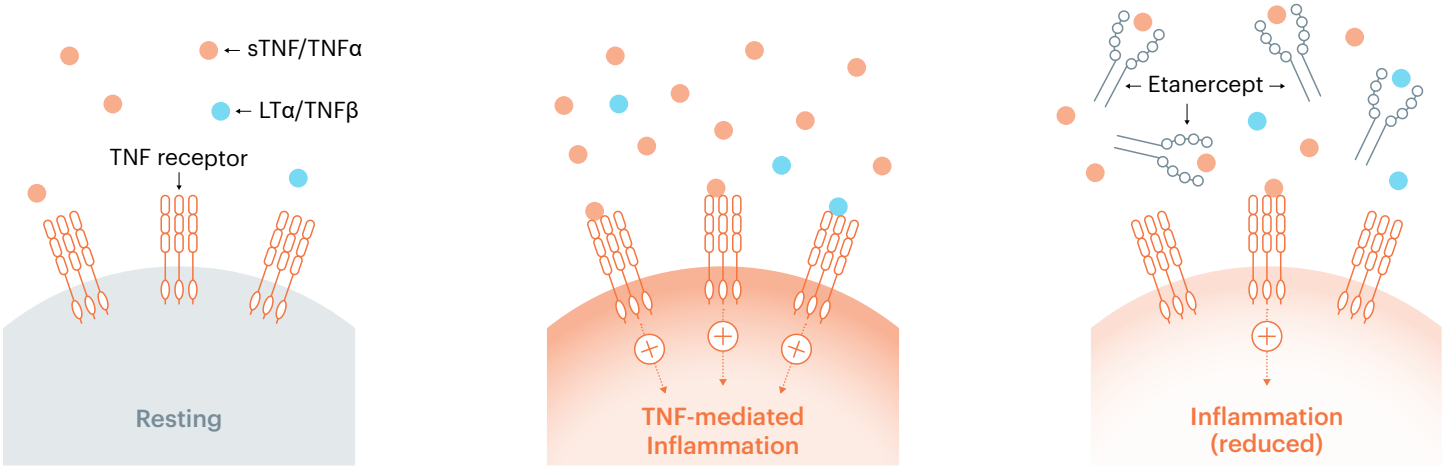
Introduction

Etanercept is a dimeric fusion protein consisting of the extracellular ligand binding domain of human tumor necrosis factor receptor– 2 (TNFR-2) fused with the Fc domain of human IgG1. It functions as a TNF antagonist to competitively inhibit TNF from binding to its cell surface receptors, thereby neutralizing TNF's biological activity (**Figure 1**). Through this mechanism of action, etanercept has been used in the treatment of moderate to severe autoimmune diseases. Etanercept was approved in the United States in 1998 and the European Union in 2000. Several etanercept biosimilars have already been approved, with many more currently in development.

Biosimilars, such as those for etanercept, must be as safe and effective as their originator counterparts; therefore, their critical quality attributes (CQAs) should be characterized extensively to ensure comparability with originator drugs, including but not limited to, biological activities, physicochemical properties, safety and stability. To assess these attributes, reference standards and control samples are recommended to be established as early as possible to minimize risk as product development progresses. This is especially true for bioassays which are well known for their inherent variability and often pose challenges to method development and maintenance of critical reagents and in-house standards.

About the USP etanercept for bioassays reference standard

Given the need for more consistent potency assessments and to help accelerate more biosimilar approvals, USP introduced the Etanercept for Bioassays Reference Standard (RS) (catalog #1255900). The USP Etanercept for Bioassays RS is a recombinant protein consisting of 934 amino acids with post-translational modification (impacting its reported molecular weight). The material was produced in Chinese Hamster Ovary (CHO) cells and formulated in a buffer composed of 1% sucrose, 4% (w/v) mannitol, 10 mM Tris HCl, and 0.2% (w/v) human serum albumin (hint: evaluate the compatibility of the formulation buffer with the buffer used in your assay). The Etanercept for Bioassay RS is provided in a lyophilized format to ensure long-term stability. The activity



TNF: Tumor necrosis factor | sTNF: soluble TNF/TNFα | LTα = lymphotoxin α/TNFβ

Figure 1. Etanercept mechanism of action.

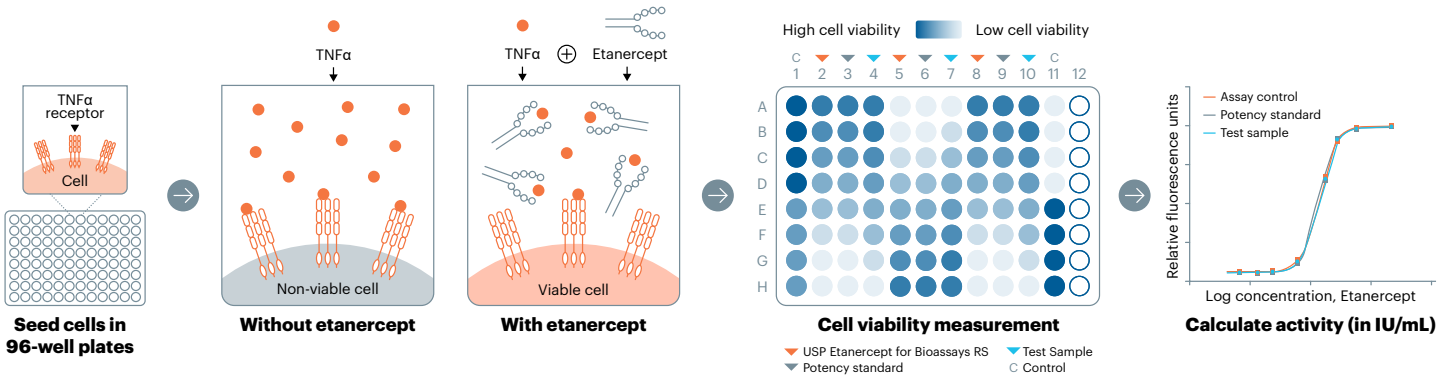


Figure 2. Workflow to assess Etanercept activity using the L929 bioassay.

was determined through a multi-laboratory global study to be 10,753 IU per ampule relative to the WHO International Standard.

How and when to use the etanercept bioassays reference standard

A neutralization assay using L929 murine fibroblast cells was used to test the activity of Etanercept (**Figure 2**; detailed procedures presented in the Supplementary section).

The Etanercept for Bioassays RS can be used as a product-specific control in bioassays for etanercept biosimilars during their manufacturing, release and stability testing, and throughout the product lifecycle. It can be used to:

- establish system suitability testing (SST) such as signal to noise ratio,
- establish the equivalence limit for assessing parallelism, and
- define the range of the bioassay during validation.

When used as an assay control, the Etanercept for Bioassays RS should be incorporated in the routine execution of the method to ensure the assay performance is well controlled between run-to-run, and day-to-day use (see **Figure 3**).

Method validation

To validate the method and ensure it performs as intended in your laboratory, use the Etanercept for Bioassays RS as an assay control to measure TNF-α neutralization and cell viability. If the experimental value meets the value in the USP certificate, the above method along with the RS can be used with confidence when testing the biosimilar.

Sample analysis

When assessing your etanercept biosimilar product, you will need the following:

- Etanercept for Bioassays RS, which will be used for system suitability testing, for example, assay control (to ensure analytical system is functioning correctly)
- A potency standard you will use to assign potency value; per regulatory guidance, this can be your in-house standards or available public standards based on a reference product.
- Test samples, which is your etanercept biosimilar drug substance, drug product, or in-process samples.

You will assign the potency value for your etanercept biosimilar based on the potency standard.

To support consistent potency assignment throughout the lifetime of your drug, the Etanercept for Bioassays RS can also be used to:

- Maintain the assay performance
  - There are various factors such as reagents, instruments, or analysts, which can result in assay variability. Having a well-characterized assay control that serves as a benchmark for data trending analysis of in-house standards allows you to assess the assay performance and identify drifts in the assay. When notable drift or changes are detected in the trending chart, an investigation may be initiated. The root cause could be attributed to a reagent, instrument, or analyst.
    - In some instances, this may necessitate further method optimization to enhance assay robustness.

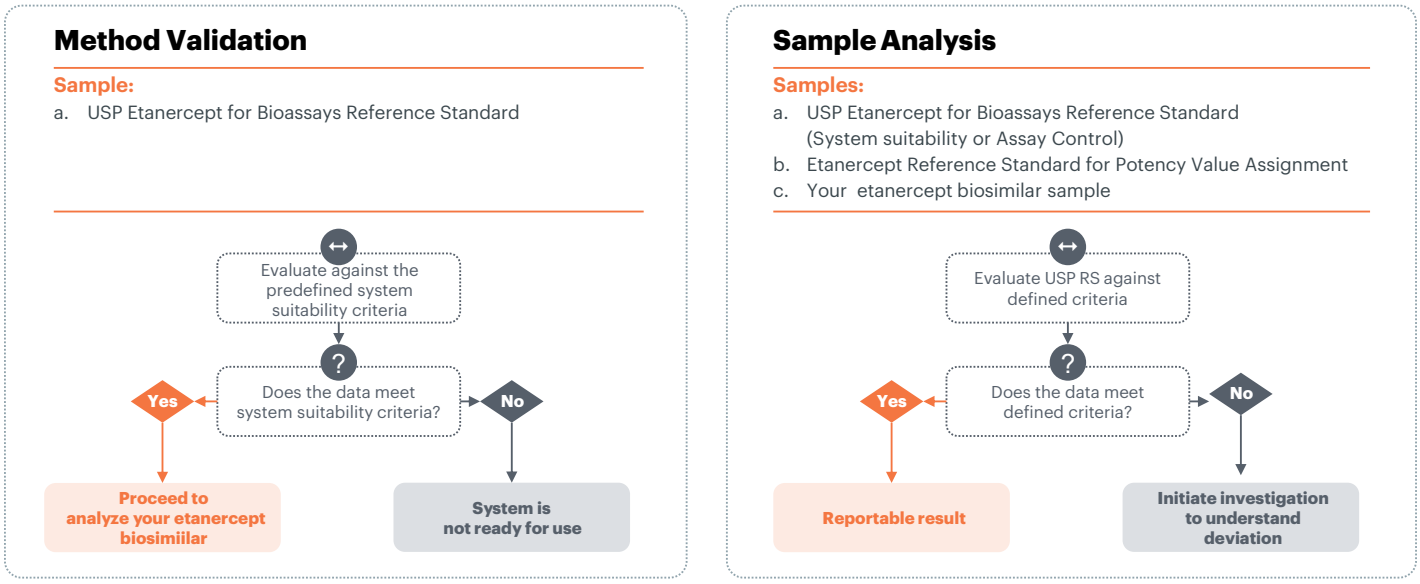


Figure 3. The Etanercept for Bioassay Reference Standard can be used to validate the method and to assess system suitability during sample analysis.

- Train personnel
  - Proper analyst training is important to deliver consistent and reliable results. The USP Etanercept for Bioassays RS value assignment is highly accurate and precise, making it suitable to be used as a sample in analyst training
- Transfer methods to other laboratories
  - Bioassays are generally the most challenging analytical technique to transfer to contract laboratories or another site. The USP Etanercept for Bioassays RS has been proven to show excellent performance and consistency in different assay platforms in multiple laboratories located globally. The implementation of this standard in the bioassay will expedite the process of analytical transfer by significantly reducing the unknown factors. This will help meet regulatory requirements and save labor, time, and cost.

Conclusion

Ensuring the biological activity of Etanercept is critical to achieve its intended therapeutic effect and safeguard patient safety. The USP Etanercept for Bioassays RS serves as a valuable tool in supporting analytical method performance during development, validation, and technology transfer. The implementation of public standards such as USP Etanercept for Bioassays RS helps standardize analytical procedures, enabling consistency of results across different laboratories. Additionally, it facilitates batch-to-batch comparability and promotes harmonization among global manufacturers.

USP maintains rigorous quality standards and routinely evaluates stability. By performing bridging tests between new and existing reference standard lots, USP standards minimize lot-to-lot variation, ensuring consistency and reliability in analytical applications. Additionally, USP standards have the advantages of being publicly available, unlike limited access to marketed products or “in-house” proprietary products. Keep in mind an in-house reference standard is required to show batch-to-batch consistency. Incorporating USP RS in product development and release testing enables greater consistency across different manufacturers and facilitates more robust assay troubleshooting and assay transfer, ultimately helping reduce analytical risk and meeting regulatory requirements.

More information
www.usp.org/biologics/proteins
Questions: uspbiologics@usp.org
Ordering information: store.usp.org/product/1255900

Test procedures for etanercept TNF-α neutralization assay using L929 cells

Media and reagent preparation

Growth media (GM) preparation

MEME Growth Medium - MEME basal media with 10% HI FBS. For preparing 500 mL GM, add:

Media / Supplement Name	Volume (mL)
Heat inactivated Fetal Bovine Serum (HI FBS)	50
Minimum Essential Medium Eagle basal medium (MEME)	445
Penicillin streptomycin solution (100X)	5

After addition of components, vacuum filter through 0.22 µm sterile filter and transfer to sterile bottle.

Assay media (AM) preparation

Assay medium - MEME basal media with 3% HI FBS.
For preparing of 500 mL AM add:

Media / Supplement	Volume (mL)
Minimum Essential Medium Eagle basal medium	485
Heat Inactivated (HI) Fetal Bovine Serum	15

After addition of all components of media, vacuum filter through 0.22 µm sterile filter and transfer to sterile reagent bottle.

TNF-α reconstitution

Reconstitute and store TNF-α as per instructions given in respective product data sheet.

Actinomycin-D reconstitution

Reconstitute actinomycin-D as per instructions given in respective product data sheet.

Resazurin solution

1. Dissolve resazurin in Dulbecco's phosphate buffered saline (DPBS) (pH 7.4) to 0.15 mg/ml.
2. Filter-sterilize through a 0.2 µm filter.
3. Store the resazurin solution protected from light at 4°C and use within 3 weeks.
4. Pre-warm to RT before use.

Procedure

Cell preparation

1. The L929 flask used for the bioassay should be 70-90% confluent (monitor the harvesting densities to address the confluency interpretation across analysts).
2. Discard spent media from the flask and wash cell monolayer with 5 - 10 mL of PBS solution.
3. Add 1 - 1.5 mL of trypsin-EDTA to the flask and incubate at 37°C for 1 - 2 mins. to allow the cell monolayer to detach from the flask surface.
4. Add 5 - 10 mL of AM to each flask to neutralize the trypsin activity.
5. Transfer the cell suspension to a sterile centrifuge tube and centrifuge at 125 x *g* for 5 min.
6. Discard the supernatant and resuspend the cell pellet in pre warmed AM.
7. Gently pipette the cell suspension up and down a few times to form uniform cell suspension.
8. Determine the cell count and % viability using trypan blue dye. Cell viability should be >90%.

Seeding assay plate

1. Adjust the live cell count to 0.75 – 0.85 $\times 10^6$ L929 cells/mL with AM.
2. Add 50 µL of 0.75 – 0.85 $\times 10^6$ L929 cells/mL cell suspension to designated wells in the assay plate and incubate cell seeded plates in a humidified CO_2 incubator for 45 minutes – 1 hour at 37 ± 1°C.

Dilution scheme and assay procedure for USP etanercept for bioassays RS

Reconstitute the USP Etanercept for Bioassays RS per the Certificate and further dilute with assay media (AM) to 120 ng/mL (6X) solution stock. This serves as a main stock for further serial dilution.

	Volumes	Total volume	Fold dilution	6X concentration (ng/mL)	Final concentration (ng/mL)
1	500 µL of USP RS from stock (120 ng/mL)	500	NA	120	20
2	100 µL of 120 ng/mL USP RS + 300 µL of AM	400	4	30	5
3	200 µL of 30 ng/mL USP RS + 200 µL of AM	400	2	15	2.5
4	200 µL of 15 ng/mL USP RS + 200 µL AM	400	2	7.5	1.25
5	200 µL of 7.5 ng/mL USP RS + 200 µL AM	400	2	3.75	0.625
6	200 µL of 3.75 ng/mL USP RS + 200 µL AM	400	2	1.875	0.3125
7	200 µL of 1.875 ng/mL USP RS + 200 µL AM	400	2	0.938	0.1563
8	100 µL of 0.938 ng/mL USP RS + 300 µL AM	400	4	0.234	0.0390

1. Add 100 µL of each of the different dilutions of the RS solution and assay control solution to the wells designated as RS and assay control in a separate plate labeled as neutralization plate. Representative plate layouts at the end of the protocol.
2. Add 100 µL of AM to the wells designated as TNF-α control wells in the neutralization plate.
3. Dilute TNF-α in AM to a final concentration of 20 IU/mL (6X stock solution is 120IU).
4. Add 100 µL of diluted TNF-α to each RS and assay control dilutions and TNF-α control wells in the neutralization plate and mix 2-3 times using micropipette. Assay control and TNF-α mix, RS and TNF-α mix are referred as neutralization mix.
5. Incubate the neutralization plate at 37 ± 1°C for 1 hr in a humidified CO_2 incubator with 5 ± 0.5% CO_2.
6. After incubation, transfer 50 µL of above neutralization mix to designated wells in the assay plate. Transfer 50 µL of TNF-α from neutralization plate to TNF-α control wells in the assay plates. Refer to the plate suggested plate map for better understanding.
7. Dilute actinomycin D with AM to achieve a final concentration of 4 µg/mL (3X stock solution is 12 µg/mL).
8. Add 50 µL of actinomycin D to wells designated for the standard and assay control in the assay plate.
9. Add 50 µL of AM to TNF-α control wells and 100 µL of AM to cell control wells in the assay plate.
10. Incubate the assay plate for 18-22 hr in a humidified incubator at 37 ± 1°C & 5 ± 0.5% CO_2.
11. Add 20 µL of pre-warmed resazurin to each well and incubate at 37 ± 1°C & 5 ± 0.5% CO_2 for an additional 6-8 hr.
12. After incubation, allow the plates to cool to room temperature (for ~10–15 min)
13. Measure the fluorescence using a suitable 96 well plate reader at excitation wavelength of 530 nm and emission wavelength of 590 nm.

Data analysis

a. Generate dose response curves using the final concentrations (concentrations before the addition of resazurin) of USP Etanercept for Bioassays RS.

b. When using as an assay control, calculate relative potency of the assay control against suitable reference standard using a suitable parallel curve method. Trend the relative potencies, fold response (highest to lowest concentration response), replicate variation (%CV) to monitor the assay performance.

For further information, please refer to *USP-NF* General Chapters:

- *<1032> Design and Development of Biological Assays*
- *<1033> Biological Assay Validation*
- *<1034> Analysis of Biological Assays*

System suitability criteria

a. The ratio between the relative fluorescent unit (RFU) of highest and lowest concentration of the dose response range of the standard solutions is NLT 3.

b. %CV of replicate variation (RFU) should be NMT 20%

Recommended plate layouts – Randomized design

Plate 1

		Assay control (USP RS) (ng/mL)	Reference Standard (ng/mL)	Sample (ng/mL)	Assay control (USP RS) (ng/mL)	Reference Standard (ng/mL)	Sample (ng/mL)	Assay control (USP RS) (ng/mL)	Reference Standard (ng/mL)	Sample (ng/mL)	Control (ng/mL)	
	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	20	20	20	20	20	20	20	20	20	TNF α control	PBS
B	PBS	5	5	5	5	5	5	5	5	5	TNF α control	PBS
C	PBS	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	TNF α control	PBS
D	PBS	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	TNF α control	PBS
E	PBS	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625	Cell control	PBS
F	PBS	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	Cell control	PBS
G	PBS	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	Cell control	PBS
H	PBS	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	Cell control	PBS

Plate 2

		Sample (ng/mL)	Reference Standard (ng/mL)	Assay control (USP RS) (ng/mL)	Sample (ng/mL)	Reference Standard (ng/mL)	Assay control (USP RS) (ng/mL)	Sample (ng/mL)	Reference Standard (ng/mL)	Assay control (USP RS) (ng/mL)	Control	
	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	0.0390	Cell control	PBS
B	PBS	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	0.1563	Cell control	PBS
C	PBS	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	0.3125	Cell control	PBS
D	PBS	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625	Cell control	PBS
E	PBS	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	TNF α control	PBS
F	PBS	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	TNF α control	PBS
G	PBS	5	5	5	5	5	5	5	5	5	TNF α control	PBS
H	PBS	20	20	20	20	20	20	20	20	20	TNF α control	PBS

Plate 3

		Sample (ng/mL)	Reference Standard (ng/mL)	Assay control (USP RS) (ng/mL)	Sample (ng/mL)	Reference Standard (ng/mL)	Assay control (USP RS) (ng/mL)	Sample (ng/mL)	Reference Standard (ng/mL)	Assay control (USP RS) (ng/mL)	Control	
	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	20	20	20	0.0390	0.0390	0.0390	20	20	20	Cell control	PBS
B	PBS	5	5	5	0.1563	0.1563	0.1563	5	5	5	Cell control	PBS
C	PBS	2.5	2.5	2.5	0.3125	0.3125	0.3125	2.5	2.5	2.5	Cell control	PBS
D	PBS	1.25	1.25	1.25	0.625	0.625	0.625	1.25	1.25	1.25	Cell control	PBS
E	PBS	0.625	0.625	0.625	1.25	1.25	1.25	0.625	0.625	0.625	TNF α control	PBS
F	PBS	0.3125	0.3125	0.3125	2.5	2.5	2.5	0.3125	0.3125	0.3125	TNF α control	PBS
G	PBS	0.1563	0.1563	0.1563	5	5	5	0.1563	0.1563	0.1563	TNF α control	PBS
H	PBS	0.0390	0.0390	0.0390	20	20	20	0.0390	0.0390	0.0390	TNF α control	PBS