DetectX® TNF-alpha Mouse
ELISA Kit

1 Plate Kit – Catalog No. K091-H1

Sample Types Tested:

Serum, EDTA Plasma, Heparin Plasma, Tissue Culture Media

Please read this insert completely prior to using the product. For research use only.
Not for use in diagnostic procedures.
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TABLE OF CONTENTS

SUPPLIED COMPONENTS & STORAGE

		K091-H1	Description
Goat anti-Mouse TNF-alpha Clear Coated 96-well Plate	Quantity	1	Strip well 96-well plate coated with goat anti-mouse TNF-alpha
	Catalog No.	C323-1EA	
Mouse TNF-alpha Standard	Quantity	2	Recombinant mouse TNF-alpha lyophilized at 1,800 pg/vial
	Catalog No.	C324-1EA	
DetectX® Biotinylated Mouse TNF-alpha Antibody	Volume	11 mL	Ready-to-use biotinylated antibody specific to mouse TNF-alpha
	Catalog No.	C325-11ML	
DetectX® Streptavidin-Peroxidase Conjugate	Volume	11 mL	Ready-to-use Streptavidin-HRP conjugate in stabilizing solution
	Catalog No.	C326-11ML	
Assay Buffer Concentrate 5X	Volume	28 mL	5X concentrate that must be diluted
	Catalog No.	X065-28ML	
Wash Buffer Concentrate 20X	Volume	2 x 30 mL	20X concentrate that must be diluted
	Catalog No.	X007-30ML	
TMB Substrate	Volume	11 mL	3,3',5,5'-Tetramethylbenzidine, a substrate for HRP
	Catalog No.	X019-11ML	
Stop Solution	Volume	5 mL	1M solution of hydrochloric acid CAUSTIC
	Catalog No.	X020-5ML	
Plate Sealer	Quantity	3	-
	Catalog No.	X002-1EA	

The unopened kit must be stored at -20°C.

Once opened, the kit can be stored at 4°C until the expiration date on the kit label, except for the Mouse TNF-alpha Standard (C324-1EA) and DetectX® Biotinylated Mouse TNF-alpha Antibody (C325-11ML) which must be stored at -20°C.

For best practice, place small volume aliquots of the antibody in high-quality polypropylene tubes prior to re-freezing to avoid multiple freeze-thaw cycles.

OTHER MATERIALS REQUIRED

- Distilled or deionized water
- Adjustable pipettes with disposable tips capable of dispensing 25 µL, 50 µL, and 100 µL. Repeater pipette or multichannel pipettes with corresponding tips are also recommended.
- Glass or high-quality polypropylene test tubes for standard and sample preparation
- An orbital microplate shaker
- A plate reader capable of measuring absorbance at 450 nm
- Software for converting optical density (OD) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.
- Optional: automated plate washer. Refer to Plate Washing Instructions for more details.
    - https://bit.ly/3tBT7N4

PRECAUTIONS

- Read this insert completely prior to using the product.
- This kit may not perform as described if any reagent or procedure is replaced or modified. Do not interchange reagents from different kit lots.
- Take appropriate safety precautions, such as: avoid breathing fumes, wear personal protective equipment (gloves, clothing, eye and face protection), and familiarize yourself with SDS documents.
    - https://www.ArborAssays.com/documentation/msds/K091-H1_MSDS.pdf
- Ensure all buffers used for samples are azide free and that any plate washing system is rinsed well with deionized water prior to using the supplied Wash Buffer. Buffers, including other manufacturers' wash buffers, that contain sodium azide will inhibit color production from the enzyme.
- **Take appropriate precautions when handling the Stop Solution, which is a caustic acid.**

BACKGROUND

TNF-alpha is the prototypic pro-inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signaling events within cells.[1] TNF-alpha is also important for resistance to infection and cancers.[2] It is central to host defense and inflammatory responses but under certain circumstances also triggers necrosis or apoptosis. Its pleiotropic effects often lead to opposing outcomes during the development of immune-mediated diseases such as cancer, Alzheimer's, inflammatory bowel disease, Crohn's Disease, ulcerative colitis, rheumatoid arthritis, psoriatic arthritis, psoriasis, and noninfectious uveitis.[3,4,5] As a result, a number of TNF-alpha therapies and biologic agents have been FDA approved.[6,7]

ASSAY PRINCIPLE

The DetectX® TNF-alpha Mouse ELISA Kit is designed to quantitatively measure mouse TNF-alpha present in serum, plasma, and tissue culture media. The kit is a sandwich ELISA with total incubation time under 4 hours.

A recombinant mouse TNF-alpha standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with a polyclonal antibody to capture TNF-alpha present in the sample. After a 2-hour incubation, the plate is washed. A biotinylated TNF-alpha antibody is added and the plate is incubated for an additional 60 minutes. Following a second wash, peroxidase-conjugated streptavidin is added, and the plate is incubated for 30 minutes and washed. Substrate is then added to the plate, which reacts with the bound peroxidase-conjugated streptavidin. After an incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450 nm wavelength. The concentration of the TNF-alpha in the sample is calculated, after making suitable correction for dilution, using software available with most plate readers.

REAGENT PREPARATION

Except for the reagents listed below, all kit components are ready for use.

Reagent	Preparation	Stability
1X Assay Buffer	Warm 5X Assay Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 5X Assay Buffer Concentrate with 4 volumes deionized water.	1X Assay Buffer is stable for 3 months at 4°C
1X Wash Buffer	Warm 20X Wash Buffer Concentrate to room temperature and mix thoroughly by inversion. Mix 1 volume 20X Wash Buffer Concentrate with 19 volumes deionized water.	1X Wash Buffer is stable for 3 months at room temperature
Standard 1	Add 600 µL 1X Assay Buffer to one vial of lyophilized Mouse TNF-alpha Standard (C324-1EA). Incubate for 5 minutes at room temperature, and then vortex briefly to mix.	Use within 2 hours of reconstitution

SAMPLE PREPARATION

For samples containing particulates, centrifuge prior to use.

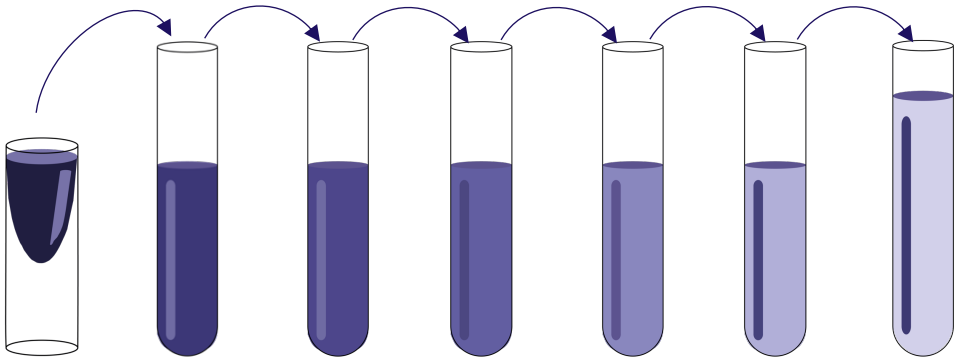
Sample Type	Procedure
Serum[+]	• Minimize the time needed during clotting and centrifugation. • Prepare a minimum 2-fold dilution of sample by adding 250 µL serum to 250 µL 1X Assay Buffer. • Samples may require further dilution with 1X Assay Buffer to fall within the standard curve range.
Plasma[+#] (K2 EDTA, Lithium Heparin)	• Keep samples on ice and process immediately. • Prepare a minimum 2-fold dilution of sample by adding 250 µL plasma to 250 µL 1X Assay Buffer. • Samples may require further dilution with 1X Assay Buffer to fall within the standard curve range.
Tissue Culture Media[+] (TCM)	• This assay has been validated using RPMI-1640 with and without 10% FBS. Other types of TCM should be validated before use. • Samples should be diluted in TCM and read off a standard curve generated in the same TCM. • Alternatively, prepare a minimum 2-fold dilution of sample by adding 250 µL TCM to 250 µL 1X Assay Buffer. • Samples may require further dilution with TCM or 1X Assay Buffer to fall within the standard curve range.

+ Use caution when preparing samples as to avoid activating TNF-alpha, which can result in high sample background.
# Sodium Citrate Plasma is not suitable for use in this assay

⚠ **Use all samples within 2 hours of dilution, or store at -20°C until ready to perform assay.**

STANDARD PREPARATION

1. Prepare Standard 1 as indicated in the Reagent Preparation section.
2. Label tubes Standard 2 through 7.
3. Add 350 µL 1X Assay Buffer to Standard 2 – 7 tubes.
4. Transfer 250 µL of Standard 1 into Standard 2 tube to make a 2.4-fold dilution. Vortex thoroughly.
5. Transfer 250 µL of the mixed solution from Standard 2 into Standard 3 tube to make a 2.4-fold dilution. Vortex thoroughly.
6. Continue serially diluting into the remaining tubes. This process and the final concentrations are summarized in the table below.



	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7
1X Assay Buffer (µL)	600	350	350	350	350	350	350
Addition	---	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Volume of Addition (µL)	---	250	250	250	250	250	250
Final Concentration (pg/mL)	3,000	1,250	521	217	90.4	37.7	15.7

⚠ **Use all Standards within 2 hours of dilution.**

ASSAY PROTOCOL

Before You Begin:

- **Ensure all reagents have been warmed to room temperature.**
- **Dilute samples as described in Sample Preparation.**
- **Run all standards and samples in duplicate.**
- Use the blank plate template to on the back page of this booklet to design your plate layout and aid in proper sample and standard identification.
- Be sure to shake the plate as directed. Failing to shake the plate or altering the shaking speed during incubations will result in decreased signal.
- Set plate parameters on the plate reader for a 96-well Corning CoStar 2592 plate. See ArborAssays.com for plate dimension data.
- Determine the number of well strips to be used and return unused well strips to foil pouch with desiccant. Seal the foil pouch and store at 4°C. Desiccant color will change from blue to pink if the foil pouch is not properly sealed.
- If you are using only part of a strip well plate, at the end of the assay discard the used wells and retain the plate frame for use with the remaining unused wells.

1. Add 100 µL of Samples or Standards into duplicate wells.
2. Add 100 µL of 1X Assay Buffer into duplicate Zero Standard wells.
3. Cover the plate with a plate sealer and shake at room temperature at 700-900 rpm for **2 hours**.
4. Remove the plate sealer, aspirate the plate, and wash each well 6 times with 400 µL 1X Wash Buffer. Tap the plate dry on clean absorbent towels.
5. Add 100 µL of DetectX® Biotinylated Mouse TNF-alpha Antibody to each well.
6. Cover the plate with plate sealer and shake at room temperature at 700-900 rpm for **1 hour**.
7. Remove the plate sealer, aspirate the plate, and wash each well 6 times with 400 µL 1X Wash Buffer. Tap the plate dry on clean absorbent towels.
8. Add 100 µL of DetectX® Streptavidin-Peroxidase Conjugate to each well.
9. Cover the plate with the plate sealer and shake at room temperature at 700-900 rpm for **30 minutes**.
10. Remove the plate sealer, aspirate the plate, and wash each well 6 times with 400 µL 1X Wash Buffer. Tap the plate dry on clean absorbent towels.
11. Add 100 µL TMB Substrate to each well.
    - ❖ The substrate solution will begin to turn blue.
12. Incubate at room temperature for **15 minutes** without shaking.
13. Add 50 µL Stop Solution to each well.
    - ❖ The substrate solution will begin to turn yellow.
14. Read the optical density at 450 nm within 10 minutes.

CALCULATION OF RESULTS

Follow the instructions below, or use this online tool: https://www.myassays.com/assay.aspx?id=1527

1. Use four-parameter logistic curve (4PLC) software to calculate the TNF-alpha concentration for each sample. Gather all raw data OD readings from each Sample and Standard, including the Zero Standard.
   - The sample OD readings will be used, together with the 4PLC fit of the standard OD readings and known TNF-alpha concentrations, to indicate the concentration of TNF-alpha in the sample.
2. Average the duplicate OD readings for each sample and standard (Mean OD).
3. Plot the standard curve with Mean OD for the standards on the y-axis and TNF-alpha concentration (pg/mL) on the x-axis. Perform a 4PLC fit.
4. Use the sample Mean OD readings and the 4PLC fit to calculate TNF-alpha concentrations in diluted samples. If diluted sample TNF-alpha concentrations are outside of the range of the standards, the sample should be prepared again at a more appropriate dilution.

EXAMPLE:

Sample	Mean OD	Diluted Sample TNF-alpha Concentration (pg/mL)
Sample 1	0.57	792

5. Multiply the diluted sample TNF-alpha concentration by the sample dilution factor (as done in Sample Preparation) to determine the concentration of TNF-alpha in the original sample.

EXAMPLE:

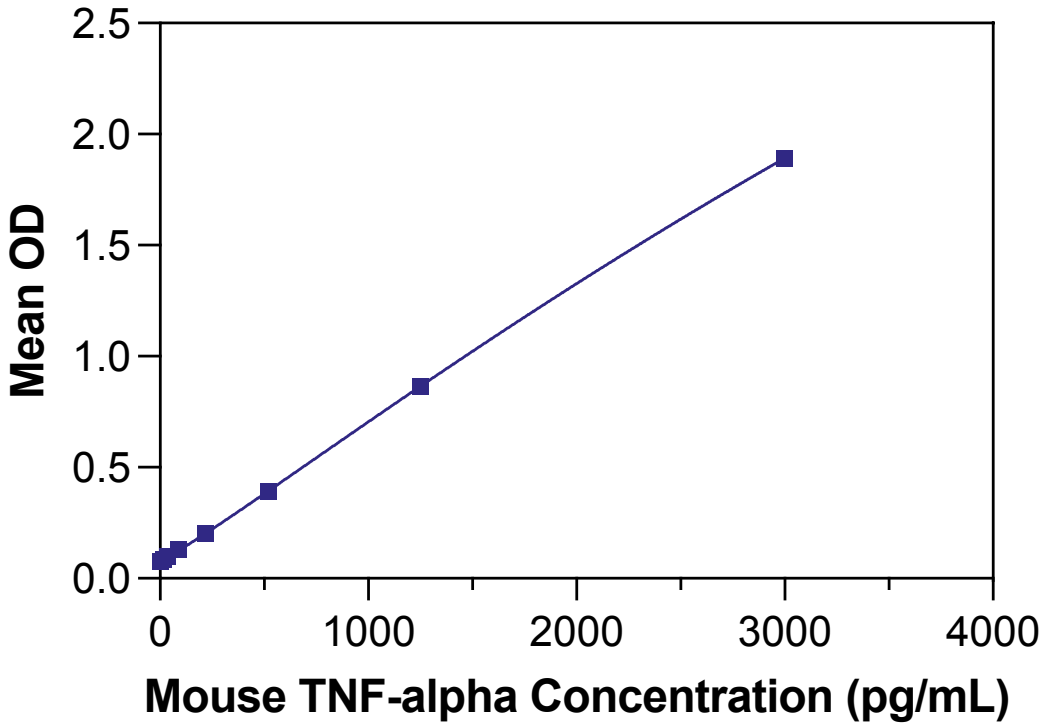
Sample	Diluted Sample TNF-alpha Concentration (pg/mL)	Sample Dilution Factor	Original Sample TNF-alpha Concentration (pg/mL)
Sample 1	792	4	3,168

TYPICAL DATA

⚠ **Always run your own standard curve. This data should NOT be used to interpret results.**

Sample	Mean OD	Diluted Sample TNF-alpha Concentration (pg/mL)	Sample Dilution Factor	Original Sample TNF-alpha Concentration (pg/mL)
Zero Standard	0.076	0	-	-
Standard 1	1.892	3,000	-	-
Standard 2	0.866	1,250	-	-
Standard 3	0.391	520.8	-	-
Standard 4	0.202	217.0	-	-
Standard 5	0.130	90.4	-	-
Standard 6	0.099	37.7	-	-
Standard 7	0.086	15.7	-	-
Sample 1	1.12	1,667	4	6,668
Sample 2	0.57	792	4	3,168

Typical Standard Curve



VALIDATION DATA

Sensitivity and Limit of Detection

Sensitivity was calculated by comparing the ODs for nineteen wells run for each of the Zero Standard and Standard #7 (minimal standard binding). The detection limit was determined at two standard deviations from the Zero Standard along the standard curve.

Sensitivity was determined as 11.0 pg/mL.

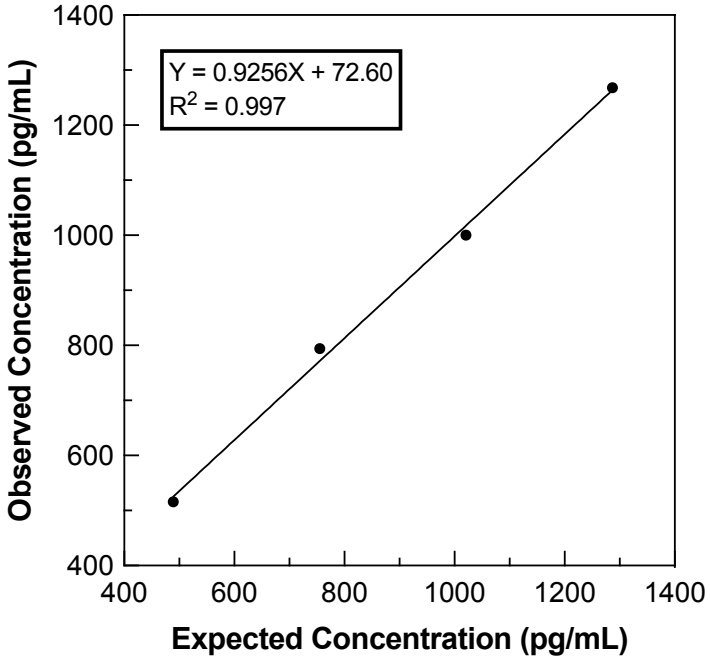
The Limit of Detection was determined in a similar manner by comparing the ODs for twenty wells run for each of the Zero Standard and a low concentration sample.

The Limit of Detection was determined as 9.3 pg/mL.

Linearity

Linearity was determined by diluting two serum samples spiked with mouse TNF-alpha. One sample had a TNF-alpha concentration of 223.2 pg/mL (low serum), and one had a TNF-alpha level of 1,552.4 pg/mL (high serum). The two samples were mixed in the ratios given below, and the measured concentrations were compared to the expected values for each given ratio.

Low Serum	High Serum	Expected Concentration (pg/mL)	Observed Concentration (pg/mL)	% Recovery
80%	20%	489	516	105.6
60%	40%	755	794	105.2
40%	60%	1,021	1,000	98.0
20%	80%	1,287	1,268	98.5
			Mean Recovery	**101.8**



Intra Assay and Inter Assay Precision

For intra assay precision, three serum samples spiked with mouse TNF-alpha were diluted in 1X Assay Buffer and 20 replicates were run in one assay. For inter assay precision, three serum samples spiked with mouse TNF-alpha were diluted in 1X Assay Buffer and duplicates of each sample were run in twenty assays run over multiple days by multiple operators. %CV represents the variation in concentration (not optical density) as determined using a reference standard curve.

	Intra Assay Precision		Inter Assay Precision	
Sample	TNF-alpha Concentration (pg/mL)	% CV	TNF-alpha Concentration (pg/mL)	% CV
1	1,738	3.5	1,694	7.4
2	1,262	4.4	1,230	7.3
3	755	3.1	740	7.7

SAMPLE VALUES

Sample pools were spiked with mouse TNF-alpha and the percent spiked recovery was determined from the recommended minimum dilution into 1X Assay Buffer.

Sample Type	Recommended Minimum Dilution	Average Recovery	Recovery Range
Serum	1:2	107.9%	100.1 – 114.4%
Citrate Plasma	Do Not Use	n/a	n/a
EDTA Plasma	1:2	99.8%	87.2 – 104.7%
Heparin Plasma	1:2	89.0%	85.5 – 115.4%
RPMI-1640	1:1.1	93.2 %	90.0 – 96.4%
RPMI-1640 + 10% FBS	1:2	88.7%	84.1 – 92.0%

INTERFERENCE

Potentially interfering substances were evaluated in the assay at both high and low concentrations of TNF-alpha.

Interferent	Effect at High TNF-alpha Concentration	Effect at Low TNF-alpha Concentration
DMSO (1%)	5.2% decrease in signal	7.2% decrease in signal
Hemoglobin (40 mg/dL)	6.6% increase in concentration	9.8% increase in concentration
Bilirubin (10 mg/dL)	14.9% decrease in concentration	5.3% decrease in concentration
Bilirubin (1 mg/dL)	5.1% decrease in concentration	No significant effect

CROSS REACTIVITY

The following cross reactants were tested in the assay at 5x, 0.5x, and 0.05x concentration of the highest standard. Percent cross-reactivity was calculated comparing observed concentration to actual concentration of each cross reactant.

Species	Antigen	Cross Reactivity (%)
Mouse	TNF-alpha	100
Human	TNF-alpha	0.1
Bovine	TNF-alpha	< 0.1
Canine	TNF-alpha	< 0.1
Cynomolgus Macaque	TNF-alpha	< 0.1
Ovine	TNF-alpha	< 0.1
Rabbit	TNF-alpha	< 0.1
Swine	TNF-alpha	< 0.1

TROUBLESHOOTING

Issue	Possible Cause & Solution
Reagent Shortage	• Check under the cap for additional reagent. Pulse spin reagent containers to collect contents prior to opening when possible. • When using a multichannel pipette, return unused reagent to container for later use.
Erratic Values	• Ensure the assay plate has been properly blotted after assay washes to remove residual wash buffer. • Prerinse pipet tips with desired reagent prior to aspirating the required volume. • Deliver volume with care to prevent splashing into adjacent wells.
High Background	• Ensure assay plate has been properly washed with the number of washes indicated in the protocol. • Reagent contamination during assay setup.
Low Signal	• Confirm tools, equipment, reagents, and containers used do not contain any trace of sodium azide. • Altering the shaking speeds or excluding shaking during incubation steps. • Verify the plate reader wavelength is 450 nm.

CITATIONS

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RELATED PRODUCTS

Kits	Catalog No.
2',3'-Cyclic GAMP ELISA and FRET Detection Kits	K067-H1/H5 K081-F1
3',3'-Cyclic GAMP ELISA Kits	K073-H1/H5
Allopregnanolone ELISA Kits	K061-H1/H5
Arg8-Vasopressin (AVP) Colorimetric and Chemiluminescent ELISA Kits	K049-H1/H5 K049-C1/C5
Atrial Natriuretic Peptide (ANP) ELISA Kits	K071-H1/H5
C-Reactive Protein (CRP) Human ELISA Kits	K069-H1/H5
Cyclic GMP Direct ELISA Kits	K065-H1/H5
DNA Damage ELISA Kits	K059-H1/H5
Endothelin-1 ELISA Kit	K045-H1
Hydrogen Peroxide (H2O2) Detection Kits	K034-H1 K034-F1
Myeloperoxidase (MPO) Human ELISA Kit	K060-H1
Nitric Oxide (NO) Colorimetric Detection Kit	K023-H1
Prostaglandin E2 (PGE2) Multi-Format ELISA Kits	K051-H1/H5
Protein Kinase A (PKA) Colorimetric Activity Kit	K027-H1
ST2 Human ELISA Kit	K055-H1

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with assay kits and reagents for wildlife conservation research.

PLATE LAYOUT

H	G	F	E	D	C	B	A	
								1
								2
								3
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