RayBio® IQELISA Kit

Human TNFa

User Manual Version 1.0
April 7, 2023

Catalog number: IQH-TNFA

Caution:
Please read manual carefully
before starting your experiment.

RayBiotech Life, Inc.

ISO 13485 & GMP Certified

3607 Parkway Lane, Suite 200
Peachtree Corners, GA 30092
Tel: 770-729-2992, Fax: 770-206-2393
Web: www.RayBiotech.com, Email: info@raybiotech.com

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TABLE OF CONTENTS

I.	INTRODUCTION	1
II.	MATERIALS PROVIDED	1
III.	ADDITIONAL MATERIALS REQUIRED	2
IV.	REAGENT PREPARATION	3
V.	STANDARD CURVE PREPARATION	3
VI.	SAMPLE PREPARATION	3
VII.	ASSAY PROCEDURE – IMMUNOCOMPLEX	4
VIII.	ASSAY PROCEDURE – qPCR	5
IX.	ASSAY PROCEDURE SUMMARY	6
X.	CALCULATION OF RESULTS	6
XI.	ASSAY PERFORMANCE	7
XII.	TROUBLESHOOTING GUIDE	8

I. INTRODUCTION

The RayBio® **I**mmuno **Q**uantitative **E**nzyme **L**inked **I**mumuno**S**orbent **A**ssay (IQELISA) is an innovative new assay that combines the specificity and ease of use of an ELISA with the sensitivity of real-time PCR. This results in an assay that is simultaneously familiar and cutting edge and enables the use of lower sample volumes while also providing more sensitivity. The RayBio® Human TNFa IQELISA Kit is a modified ELISA assay with high sensitivity qPCR readout for the quantitative measurement of Human TNFa in serum, plasma, and cell culture supernatants. This assay employs an antibody specific for Human TNFa coated on a 96-well PCR plate. Standards and samples are pipetted into the wells and TNFa present in a sample is bound to the wells by the immobilized antibody. The wells are washed and a detection affinity molecule is added to the plates. After washing away unbound detection affinity molecule, primers and a PCR master mix are added to the wells and data is collected using qPCR. C_t values obtained from the qPCR are then used to calculate the amount of antigen contained in each sample, where lower C_t values indicate a higher concentration of antigen.

II. MATERIALS PROVIDED

The entire kit should be stored for up to 1 year at -20°C from the date of shipment. After use, standard (recombinant protein) should be stored at -20°C or -80°C (recommended at -80°C). Opened PCR plate, Detection Reagent (DNA), Master Mix, and Primers should be stored at -20°C. All other reagents may be stored for up to 1 month at 2° to 8°C. Avoid repeated freeze-thaw cycles.

ITEM	COMPONENT	Quantity
A*	TNFa Microplate	1 96-well plate
B	20X Wash Buffer Concentrate	1 bottle (25 mL)
C	TNFa Standards	2 vials
E	5X Assay Diluent	1 bottle (15 mL)
F	TNFa Detection Antibody	2 vials
G	10X Detection Reagent (DNA)	1 vial (1 mL)
I	Primer Solution	1 vial (1.5 mL)
J	Master Mix	1 vial (1.4 mL)
K	10X PCR Preparation Buffer	1 vial (1 mL)
L	10X Final Wash Buffer	1 vial (1 mL)

**The PCR plate used is a 0.2mL, non-skirted 96-well plate (ThermoFisher, cat. no.: AB0600). Please ensure compatibility with your PCR machine prior to purchase. For additional information contact technical support (techsupport@raybiotech.com).*

III. ADDITIONAL MATERIALS REQUIRED

1. Real-time PCR instrument, Bio-Rad or QuantStudio recommended.
2. Precision pipettes to deliver 1 µL to 1 mL volumes.
3. Saran wrap to cover plates during incubation.
4. Adjustable 1-25 mL pipettes for reagent preparation.
5. 100 mL and 1 liter graduated cylinders.
6. Absorbent paper.
7. Distilled or deionized water.
8. Log-log graph paper or computer and software for data analysis.
9. Tubes to prepare standard or sample dilutions.

IV. REAGENT PREPARATION

NOTE: *Bring Wash Buffer (Item B) to room temperature (18-25°C) before use. All other reagents should be thawed and kept on ice or in 4 °C until ready to use.*
NOTE: *If wash buffers contain visible crystals, mix gently (at room temperature) until dissolved.*
NOTE: *Briefly centrifuge (~1,000g) Standards (Item C) and Detection Antibody (Item F) before use.*

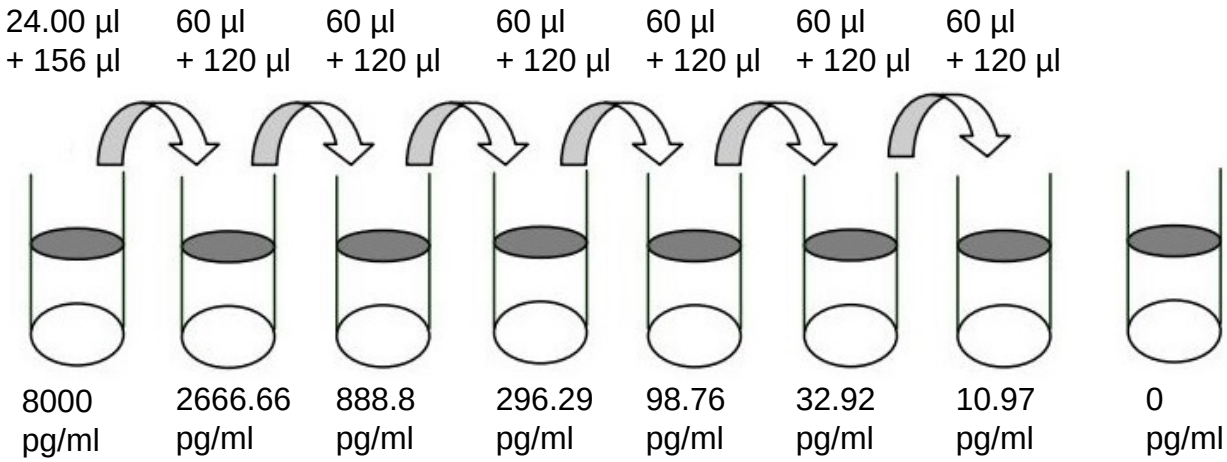
ITEM	COMPONENT	PREPARATION*	STORAGE AFTER PREPARATION
A	TNFa Microplate	No Preparation	1 year at -20°C
B	20X Wash Buffer Concentrate	Dilute 20-fold** with distilled or deionized water.	6 months at 4°C
C	TNFa Standards	See Section V for preparation and storage instructions.	
E	5X Assay Diluent	Dilute 5-fold with distilled or deionized water to prepare "1X Assay Diluent"	6 months at 4°C
F	TNFa Detection Antibody	Add 25 µl 1X Assay Diluent to prepare 80X Detection Antibody Concentrate. Dilute 80X Detection Antibody Concentrate 80-fold with 1X Assay Diluent to prepare 1X Detection Antibody.	80X Concentrate: 1 month at 4°C. 1X Detection Antibody:Use immediately. Do not store.
G	10X Detection Reagent (DNA)	Dilute 10-fold with distilled or deionized water.	Use immediately. Do not store.
I	Primer Solution	Mix equal parts primer solution and master mix immediately before use to prepare 1:1 Primer/Master Mix solution.	Use immediately. Do not store.
J	Master Mix		
K	10X PCR Preparation Buffer	Dilute 10-fold with distilled or deionized water.	6 months at 4°C
L	10X Final Wash Buffer	Dilute 10-fold with distilled or deionized water.	

**If running partial assays (e.g., half a plate), determine how much volume of Items C, F, I, and J are needed and prepare only that amount.*

***A 20-fold dilution is the same as 1-part 20X Wash Buffer Concentrate and 19-parts water.*

V.STANDARD CURVE PREPARATION

1. Add 400 µl 1X Assay Diluent into the Standard vial to prepare a 60 ng/ml working solution. Resuspend lyophilized powder by gently mixing. Store on ice.
2. Prepare the first standard dilution by pipetting 24.00 µl of the 60 ng/ml working solution into 156 µl of 1X Assay Diluent. Label this tube “Standard Dilution 1.”
3. Pipette 120 µl 1X Assay Diluent into each of six labeled tubes (Standard dilution 2 – 7).
4. Serially dilute by transferring 60 µl of the previous dilution into the subsequent tube in the dilution series.
   Note:*Mix each tube thoroughly and spin down at low speed before next transfer.*
5. 1X Assay Diluent will serve as the zero standard (0 pg/mL).



VI.SAMPLE PREPARATION

NOTE: *It is recommended that all samples be run at least in triplicate.*

NOTE: *Target protein levels may vary between different specimens. Optimal dilution factors for each sample must be determined empirically by the investigator. We recommend a minimum sample dilution of 2x to minimize matrix effects.*

1. For this kit, it is recommended to use a sample dilution of 5, 10, 20 fold.
2. Add the appropriate amount of sample to 1x Assay Diluent to achieve the desired sample dilution fold.
3. For Example: For a 10-fold dilution, add 9 µL sample to 81 µL 1X Assay Diluent. (Only 75 µL total is needed to run the sample in triplicate but it is recommended to make extra volume to allow for volume lost in transfer)
4. Store on ice and mix well before use.

VII. AASSAY PROCEDURE – IMMUNOCOMPLEX

Note: *All incubations and wash steps must be performed at room temperature under gentle rocking or rotation (~1-2 cycles/sec).*

Note: *Partial plate runs may be accomplished by cutting the PCR plate (and PCR Plate Film) into the desired number of strips using a pair of clean, sturdy scissors, wire cutters, or shears. The remainder of the plate can re-sealed and used at a later date.*

1. Prepare and store all reagents at the temperatures indicated in Section IV before use.
2. Add **25 µL** of each prepared standards and samples (see Sections V and VI, respectively) into each appropriate well and cover plate with plastic wrap. Spin at low speed for **30 seconds** to ensure sample is present at bottom of well. **Incubate for 1.5 to 2.5 hours**.
   Note: Volumes should be consistent between all wells, samples, and standards. As little as 10 µL can be used to conserve sample, but this increases the risk of technical errors.
3. Discard the solution and wash **4 times** with 1x Wash Buffer. Wash by filling each well with **100 µL** 1x Wash Buffer using a multi-channel pipette. Ensure no bubbles are present at the bottom of wells that may otherwise impair efficient washing. Dislodge any bubbles with gentle tapping. Remove Wash Buffer by aspirating or decanting. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining 1x Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add **25 µL** of prepared 1X Detection Antibody to each well, cover plate, and spin at low speed for 30 seconds. Incubate for **1 hour**.
5. Discard the solution. Repeat the wash as in step 3.
6. Add **50 µL** prepared 1X Detection Reagent (DNA) to each well, cover plate, and spin at low speed for 30 seconds. Incubate. Incubate for **1 hour**.
7. Discard the solution. Repeat the wash as in step 3.
8. Add **75 µL** prepared 1X Final Wash Buffer to each well. Dislodge any bubbles with gentle tapping. Incubate for **4 minutes** and then remove the solution from each well and blot against paper towels.
   Note: Do not exceed an incubation time of 5 minutes as this can destabilize the complex.
9. Add **75 µL** prepared 1X PCR Preparation Buffer to each well. Dislodge any bubbles with gentle tapping. Incubate for **10 seconds** and then remove the solution from each well and blot against paper towels.

VIII. ASSAY PROCEDURE – qPCR

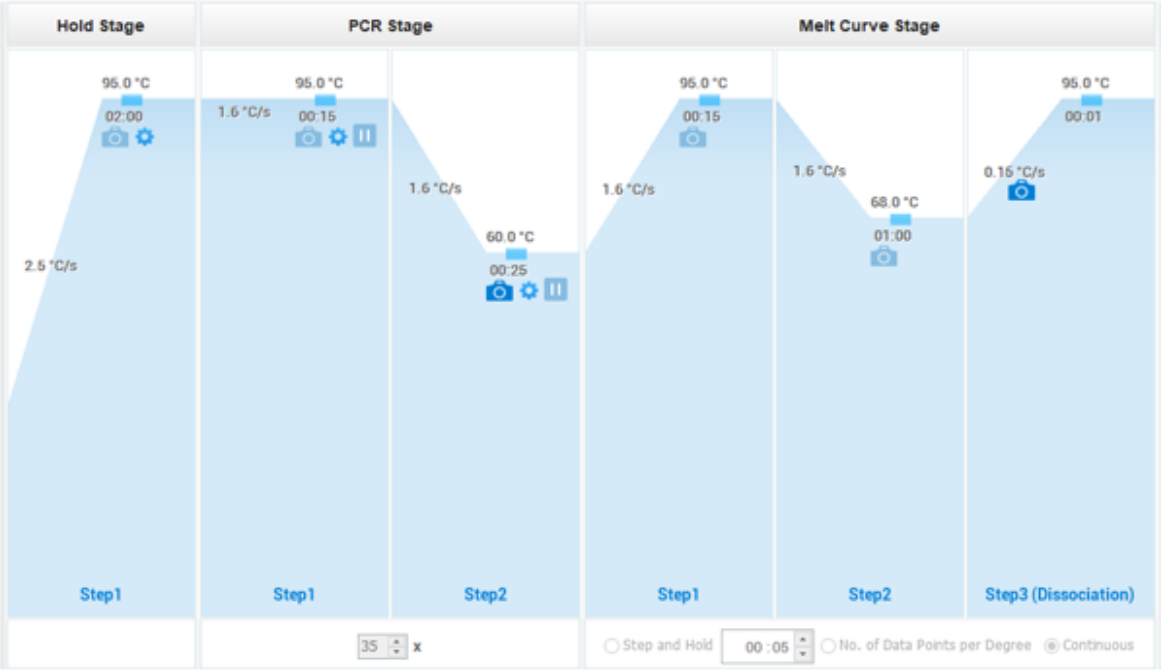
1. Prepare and store all reagents at the temperatures indicated in Section IV before use.
2. Add **20 µL** of prepared 1:1 Primer/Master Mix solution to each well of the plate.
3. Cover plate with provided PCR Plate Film. Ensure the film is completely and evenly pressed onto the plate, creating an airtight seal around each well of the plate. Spin at low speed for 30 seconds.
   Note *At this stage, the covered plate can be stored overnight at 4°C. Do not exceed 18 hours*.
4. Place the plate into a FITC compatible real-time PCR instrument for detection.
5. Set the PCR reaction conditions according to Table 1. Set the reaction volume to 20 µL.

Table 1: PCR Program

Step		Temperature	Time	Temperature Ramp Rate	Number of Cycles
Stage 1	Initial Denaturation	95ºC	2 min	2.5ºC	1
Stage 2	Denature	95ºC	15 sec	1.6ºC	35
	Annealing, extension, and fluorescence detection	60ºC⁺	25 sec	1.6ºC	
Stage 3*	Melt Curve	95ºC	15 sec	1.6ºC	1
		68ºC	1 min	1.6ºC	
		95ºC⁺	1 sec	0.15ºC	

**Stage 3 (melt curve) is optional, but recommended to view potential plate contamination that can cause high background and lower sensitivity. This can be seen in the visual aid on YouTube.*

⁺Ensure the PCR program is set to measure fluorescence at (1) step 2 of stage 2 and (2) step 3 of stage 3.



IX. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.
2. Add 25 µL standard or sample to each well.
3. Incubate 1.5 – 2.5 hours at room temperature.
4. Add 25 µL Detection Antibody to each well. Incubate 1 hour at room temperature.
5. Add 50 µL of IQELISA Detection Reagent to each well.
6. Incubate 1 hour at room temperature.
7. Add 20 µL Primer-Master Mix solution to each well.
8. Run real-time PCR.

X. CALCULATION OF RESULTS

The primary data output of the IQELISA kit is Ct values. These values represent the number of cycles required for a sample to pass a fluorescence threshold. As the DNA is amplified additional fluorescent signal is produced, with each cycle resulting in an approximate doubling of the DNA. Therefore, higher levels of DNA (directly related to the amount of antigen in the sample) result in lower Ct values.

Calculate the mean Ct for each set of triplicate standards, controls, and samples. Subtract the mean Ct value of the control from that of each of the standards and samples to obtain Delta Ct values (Ct). Plot Ct values of the standards against concentration.

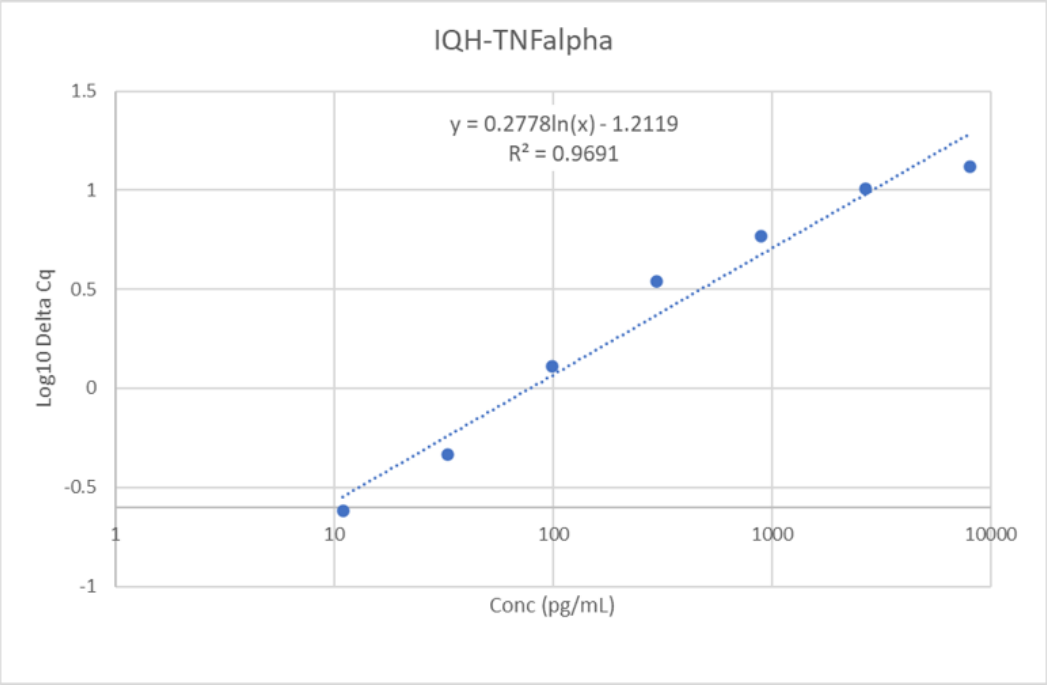
A line of best fit can then be obtained from the graphed data in one of three ways:
(1) Linear Ct vs. Linear concentration
(2) Linear Ct vs. Log concentration (semi-log)
(3) Log Ct vs. Log concentration (log-log)

The equation for the line of best fit can then be used to interpolate the concentration of the samples.

XI. ASSAY PERFORMANCE

A. TYPICAL DATA

The data are for demonstration purposes only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum quantifiable dose of TNFa is typically 16.25 pg/ml. However, concentrations below 16.25 pg/ml may be detected outside the quantification range.

C. RECOVERY

Serum spike tests show recovery is 99.27% with a range from 83% to 115%.

D. Variability

Intra-plate CV % is below 10%.

Inter-plate CV % is below 15%.

XII. TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	SOLUTION
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Briefly centrifuge standards and ensure complete resuspension
Low signal	Insufficient incubation times	Ensure sufficient incubation time. Assay procedure step 2 may be done overnight to increase signal
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV %	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
	Improper Tm	Check run parameters and calibrate instrument
Low sensitivity	Improper storage of the IQELISA kit	Store your standard at <-20°C after reconstitution, Master Mix at -20°C others at 4°C.
	Improper Tm	Check run parameters and calibrate instrument

This product is for research use only.

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