ELISA Kit Components	Amount	Part No.
TNP-coated Microwell Strip Plate	8-well strips (12)	655-101
Anti-TNP Calibrator 1 U/ml	0.65 ml	655-103-B
Anti-TNP Calibrator 4 U/ml	0.65 ml	655-103-C
Anti-TNP Calibrator 10 U/ml	0.65 ml	655-103-D
Anti-TNP Calibrator 20 U/ml	0.65 ml	655-103-E
Anti-TNP Positive Control	0.65 ml	655-103 PC
Anti-Mouse IgM HRP Conjugate (100X)	0.15 ml	H-MsM.2a11
Sample Diluent Concentrate (20X)	10 ml	SD-20T
Low NSB Sample Diluent	30 ml	TBTm
Wash Solution Concentrate (100X)	10 ml	WB-100
TMB Substrate	12 ml	80091
Stop Solution	12 ml	80101
Product Manual	1 ea	655-110-TIM

PRECAUTIONS AND SAFETY INSTRUCTIONS

Calibrators, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains dilute sulfuric acid. Follow good laboratory practices and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website.

LIMITATIONS OF THE ASSAY

Quantitation of Antibody in a Sample

The ELISA measures anti-Trinitrophenol (TNP) activity—a combination of antibody concentration and avidity for the TNP antigen. Antibodies with substantially different specific antibody concentrations may display similar anti-TNP activities due to differences in avidity. The quantitation or potency of the samples is, therefore, appropriately expressed in activity Units (titer), rather than mass units (e.g., ug/ml).

Calibrator Curve Quantitation

To quantitate antibody activity from a Calibrator curve (such as provided with the kit), the dilution curve of the samples must be parallel to the Calibrator curve, to avoid different values being obtained from different regions of the curve. Antibodies that are not matched in antigen avidity will often have non-parallel dilution curves. In these cases, antibody activity is best expressed as a titer relative to a reference positive such as the 4 U/ml Calibrator, or another Calibrator in the kit (see Calculation of Results, p. 5-6).

For more details please consult our web site (www.4adi.com) or contact us by email (service@4adi.com).

Instruction Manual No. M-655-110-TIM

Mouse Anti-Trinitrophenol (TNP) IgM

ELISA Kit Cat. #655-110-TIM

For Quantitative Determination of Anti-Trinitrophenol (TNP) IgM in Biological Fluids

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INTENDED USE

The Mouse Anti-TNP IgM ELISA Kit is an immunoassay suitable for quantifying or titering IgM class antibodies specific for TNP in serum, plasma, or other biological fluids, including tissue culture medium.

This kit is for research use only (RUO), not for diagnosis or therapeutic purposes.

RESEARCH USE OF THE TEST

Anti-Trinitrophenol (anti-TNP) antibodies are used in serological research as a defined model system for ELISAs. TNP functions as a small chemical hapten that can be readily conjugated to carrier proteins such as BSA or KLH. The simplicity and reproducibility make TNP-protein conjugates ideal antigens for ELISA development.

Anti-TNP ELISAs are valuable tools in immunology and serology research. They are used to investigate antibody isotype responses, affinity maturation, and quantitate antibody titers in immunized sera, particularly in studies examining hapten-carrier immune responses. The robustness and reproducibility of the TNP/anti-TNP system have made it a standard model in serological ELISAs, contributing to its continued use in both methodological development and use in fundamental studies of antigen-antibody interactions.

PRINCIPLE OF THE TEST

The Mouse TNP IgM ELISA kit is based on the binding of mouse anti- TNP IgM in samples to TNP-carrier immobilized on the microwells; anti- TNP IgM antibody is detected by anti-mouse IgM specific antibody conjugated to HRP (horseradish peroxidase) enzyme. After a washing step, chromogenic substrate (TMB) is added and color is developed by the enzymatic reaction of HRP on the substrate, which is directly proportional to the amount of anti-TNP IgM present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microwell reader. The amount of Mouse IgM in samples is calculated relative to a mouse anti-TNP reference antibody.

PRODUCT SPECIFICATIONS

Specificity

TNP-BSA is used to coat the microwells; thus, the assay is specific for antibodies directed to TNP and/or BSA. The anti-Mouse IgM HRP conjugate is specific for mouse IgM and does not react with IgE, IgA, or IgG class antibodies.

Assay Sensitivity

The TNP antigen coating level is optimized to differentiate anti-TNP IgM from background (non-antibody) signal with mouse serum samples diluted 1:100.

Calibrator Values

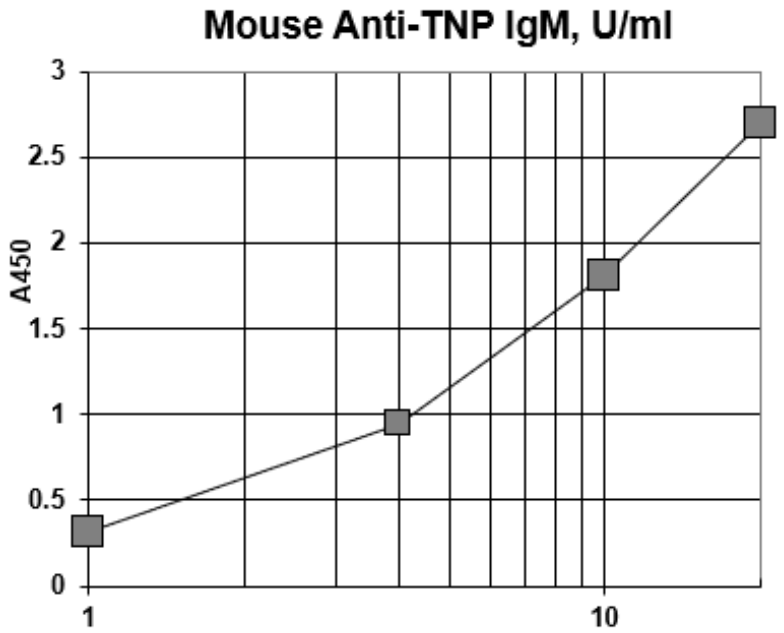
The Calibrators are composed of dilutions of an anti-TNP antibody. Values are assigned as arbitrary anti- TNP activity units (see Limits of the Assay).

CALCULATION AND IINTERPRETATION OF RESULTS (continued)

Method III. Use of a Calibrator Curve

When the dilution curves of samples are parallel to the Calibrator curve (see **Limits of the Assay**), the anti-TNP activity units may be determined by interpolation from the Calibrator curve and multiplying the value by the sample dilution (the blank OD does not need to be subtracted).

Typical Calibrator Curve



Typical Results:

Wells	Calibrators & Samples	A450 nm	U/ml
A1, A2	**Negative Diluent Control**	0.049	0
B1, B2	1 U/ml **Calibrator**	0.311	1
C1, C2	4 U/ml **Calibrator**	0.948	4
D1, D2	10 U/ml **Calibrator**	1.809	10
E1, E2	20 U/ml **Calibrator**	2.703	20
F1, F2	**Sample** [Diluted 1:100]	1.575	7.8
	Calculated: 100-fold dilution x 7.7 U/ml = **780 U/ml** in serum		

CALCULATION AND INTERPRETATION OF RESULTS

Several data reduction methods may be considered to optimize precision and to best represent the relationships among experimental and control groups.

Method I. Single Dilution or Low Titer Samples

With samples tested at a common dilution, report values in OD units, or a Calibrator value may be used as an index to normalize between-assay variation:

1. Calculate the mean net ODs for replicate samples and selected Calibrator.
2. Divide each sample OD value by the Calibrator OD value [may also multiply by sample dilution and/or Calibrator (U/ml) value] = **Activity Units.**

Typical Results: see Method III – Calibrator curve

1.02 [Sample, net OD] ÷ **0.81** [**4** U/ml Calibrator, net OD]
X **4** U/ml X **100** sample dilution = **504** U/ml.

II. Positive Index

Values of **Experimental** samples may be expressed relative to the values of **Control** samples, by calculation of a **Positive Index**. One typical method is as follows:

1. Calculate the mean + 2 SD of the Control samples = Positive Index.
2. Divide each sample value by the Positive Index. Values above 1.0 are a measure of Positive Antibody Activity; below 1.0 are negative for antibody.

Typical Results:

	ELISA OD Units		Antibody Activity	
Sample	Control	Exptl	Control	Exptl
1	0.243	2.358	0.49	**4.79**
2	0.351	0.597	0.71	**1.21**
3	0.286	1.421	0.58	**2.89**
4	0.357	1.268	0.73	**2.58**
5	0.512	0.857	**1.04**	**1.74**
6	0.342	1.296	0.70	**2.63**
7	0.298	0.608	0.61	**1.24**
8	0.285	0.369	0.58	0.75
9	0.157	0.864	0.32	**1.76**
10	0.187	0.543	0.38	**1.10**
Mean	0.302			
SD	0.095			
Mean +2 SD	**0.492**	= Positive Index		

KIT CONTENTS

The microtiter well plate and all other reagents, if unopened, are stable at 2-8ºC until the expiration date printed on the label. Stabilities of the working solutions are indicated under Reagent Preparation.

To Be Reconstituted: Store as indicated.

Component	Instructions for Use
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** and store at 2-8ºC until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume 10ml + 990ml with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at 4ºC for long term and RT for short term.
Anti-Mouse IgM - HRP Conjugate Concentrate (100x) Part: H-MsM.2a11, 0.15ml	Peroxidase conjugated anti-Mouse IgM in buffer with protein, detergents, and antimicrobial. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8ºC storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
TNP-BSA Microwell Strip Plate	655-101	8-well strips (12)	Coated with TNP-BSA, and post-coated with stabilizers.
Anti-TNP Calibrators			
1 U/ml 4 U/ml 10 U/ml 20 U/ml	655-103-B 655-103-C 655-103-D 655-103-E	0.65 ml 0.65 ml 0.65 ml 0.65 ml	Four (4) vials, each containing anti- TNP antibodies in buffer with antimicrobial.
Anti-TNP Positive Control	655-103 PC	0.65 ml	Anti-TNP diluted in buffer with protein, detergents, and antimicrobial. [Value range on label]
Low NSB Sample Diluent Reduces non-specific binding	TBTm	30 ml	Buffer with protein, detergents, and antimicrobial. Use as is for sample dilution. Not for HRP Conjugate dilution
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Dilute sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Anti-Mouse IgM-HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate and Sample Diluent concentrate; 200ml to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For serum, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. If samples will not be assayed immediately, store refrigerated for up to a few weeks or frozen for long-term storage. Avoid freeze-thaw cycles.

Antibody Stability & Dilution

Initial dilution of serum into **Working Sample Diluent** (WSD) is recommended to stabilize antibody activity. This enhances reproducible sampling and stabilizes the antibody activity for years, stored refrigerated or frozen. Further dilution into **Low NSB Sample Diluent** (LNSD), which provides the lowest assay background, should be at least 5 times the initial dilution and performed the same week as the assay.

Example: Initial (1/5): 10ul serum + 40ul WSD [or 0.1ml + 0.4ml]
Further (1/100): 10ul initial (1/5) + 190ul LNSD (1/100)

Assay Design

Review Interpretation of Results (p. 5-6) before proceeding:

- Select the proper sample dilutions accounting for expected potency of positives and minimizing non-specific binding (NSB) and other matrix effects; for example, net signal for negative samples should be lower than the **1 U/ml Calibrator**. This is usually 1:100 or greater dilution for mouse serum with normal levels of IgG and IgM.
- Run a Sample Diluent **Blank**. This signal is an indicator of proper assay performance, especially of washing efficacy, and is used for net OD calculations, if required. Blank OD should be <0.3.
- Run the Anti-TNP Positive Control; the value range is on the label.
- Run a set of **Calibrators**, which validate that the assay was performed to specifications: **20 U/ml** should give a high signal (>1.5 OD); **1 U/ml** should give a low signal which can be used to discriminate at the Positive/Negative threshold (see Interpretation of Results, p. 5-6).

ASSAY PROCEDURE

Plate Set-up

Bring all reagents to room temperature (18-30° C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 8 Calibrator wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Add 200-300ul Working Wash Solution before sample addition to each well and let stand for about 1-5 minutes. Aspirate or dump the liquid and pat dry on a paper towel.

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation [100ul – 60 min; 4 washes]

- Add 100ul of Calibrators, samples, and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 60 minutes.
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of diluted Anti-Mouse IgM-HRP Conjugate to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 1.

3. Substrate Incubation [100ul – 15 min]

- Add 100ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time or read OD at 405-410 nm (results are valid).

4. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

5. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.