Microplate Assay for
Canine Thyroglobulin Auto-Antibody (TgAA)

MBS480811
This product is sold for in vitro veterinary diagnostic use only. Not for human clinical diagnostic use.

INTENDED USE
The Microplate Assay for Canine Thyroglobulin Auto-antibody Enzyme Immunoassay (TgAA EIA) Kits are to be used for the diagnosis of autoimmune thyroid disease in dogs. Purified canine thyroglobulin (Tg) is used to detect autoantibodies to Tg. Dogs that test negative using this simple assay (product MBS480810), are free of Tg autoantibodies. However, recent studies have shown that some (less than 10%) of the positive results obtained using the MBS480810 kit alone are false positives resulting from non-specific binding of canine Ig to the test wells. Therefore, dogs that test positive should be retested with the non-specific Ig binding values subtracted (using MBS480810 and MBS480811 kits). This method eliminates false-positives and reduces inconclusive results due to non-specific Ig binding.

The MBS480811 kit contains reagents identical to those in the MBS480810 kit. However, the wells are not coated with thyroglobulin (Tg). This allows for the quantification of non-specific binding for each sample.
Alternatively, the MBS480812 kit can be used. This kit contains a single microplate containing both thyroglobulin coated (Tg+) and non-specific binding (Tg-) strips.

PRINCIPLES OF PROCEDURE
Thyroglobulin (Tg) is a 660 kD thyroid protein that occurs as a 19S dimer. During synthesis, both monomers appear to be identical, but are heterogeneous following glycosylation and iodination. Tg is the major storage form for thyroxine and triiodothyronine in the thyroid follicle. Tg has many epitopes, as anticipated for a protein of its size. Monoclonal antibodies with multiple specificities have been developed against human Tg, and the reactivity of some may be inhibited by T4 but not by other iodoamino acids. Several studies have shown the presence of autoantibodies (AA) to Tg in dogs to be an indicator of lymphocytic thyroiditis. This disease is comparable, histologically, to Hashimoto's thyroiditis in humans. Evidence suggests that autoimmune thyroiditis is due to a genetic defect in humans and dogs.

This indirect enzyme immunoassay is for the determination of Tg autoantibodies in canine serum. Purified canine Tg is coated on the wells of a 96-well polystyrene plate. Canine test sera, along with positive and negative canine control sera are added to the plate and allowed to bind. Next, the enzyme labeled anti-immunoglobulin is then added and autoantibodies are allowed to incubate and bind. The enzyme substrate is then added, and the plate is incubated. The higher the concentration of TgAA in the serum sample, the higher amount of substrate bound and the brighter the color. The reaction is terminated by the addition of a stop solution.

A schematic diagram showing how this enzyme immunoassay works is presented below:

These assays are not intended for human samples or for in vitro diagnosis of human subjects.

*FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES*
REAGENTS SUPPLIED

<table>
<thead>
<tr>
<th>Component</th>
<th>Description</th>
<th>Volume</th>
<th>Storage</th>
<th>Cat. No.</th>
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<tr>
<td>Positive Ref. Serum</td>
<td>TgAA Positive Reference Serum</td>
<td>50 µL</td>
<td>2-8 °C</td>
<td>NA</td>
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<tr>
<td>Negative Ref. Serum</td>
<td>TgAA Negative Reference Serum</td>
<td>50 µL</td>
<td>2-8 °C</td>
<td>NA</td>
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<td>Anti-Canine IgG-HRP</td>
<td>Rabbit Anti-Canine IgG-HRP Conjugate.</td>
<td>30 µL</td>
<td>2-8 °C</td>
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<td>EIA Phosphate Buffer</td>
<td>Blue buffer used to dilute reagents and samples.</td>
<td>125 mL</td>
<td>2-8 °C</td>
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<td>TMB Substrate</td>
<td>Color reagent</td>
<td>20 mL</td>
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<td>NA</td>
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<td>Wash Buffer (10x)</td>
<td>Yellow buffer used to wash the plate.</td>
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<td>Canine Tg Plate</td>
<td>96 Well Tg Coated (Tg⁺) Plate.</td>
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<td>NSB Plate</td>
<td>96 Well NSB (Tg⁻) Plate.</td>
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<td>MBS480811</td>
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<td>Tg &amp; NSB Plate</td>
<td>96 Well Combo (Tg⁺/Tg⁻) Plate.</td>
<td>1</td>
<td>2-8 °C</td>
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<tr>
<td>Reagent Troughs</td>
<td>Used for dispensing reagents.</td>
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</table>

MATERIALS NEEDED BUT NOT PROVIDED

1. Pipettors and tips: 50-200 µL multiwell pipettor; 20-200 µL and 200-1000 µL adjustable pipettor.
2. Beakers, flasks, and cylinders necessary for preparation of reagents
3. 5 mL 3N H₂SO₄ (stop reagent)
4. 96-well plate washing/ aspiration device
5. 96-well plate reader for measurement of absorbance at 450 nm
6. Deionized water

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. Do not use any components beyond stated expiration dates.
2. Do not mix reagents or components of this kit with any reagents or components of any other kit
3. It is not necessary to allow reagents to reach room temperature before use.
4. Desiccant bag must remain in foil pouch with any unused strips.
5. Always use different pipette tips for the buffer, serum, anti-IgG, TMB, and stop solution.
6. If you plan to run only a few assays at a time, it is recommended that you only prepare the needed amount of Positive Reference, Negative Reference, and HRP working solutions.
7. When pipetting into the wells, do not allow the pipette tip to touch the inside of the well, or any of the reagents already in the well as this can cause contamination.
8. Always pour TMB Substrate out of the bottle into a clean reagent trough. DO NOT pipette out of the bottle.
9. For best results, perform assays in duplicate.
10. Do not wash the plates with a wash or squirt bottle. This can cause insufficient washing, excessively harsh washing, or cross contamination.

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SUGGESTED TEMPLATES

For MBS480810 and MBS480811 Kits (42 unknowns in duplicate per kit)

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<thead>
<tr>
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Where:  
+R = Positive Reference Serum  
-R = Negative Reference Serum  
BLK = Blank wells (buffer in place of serum)  
U = Unknowns

For MBS480812 Kit (21 unknowns in duplicate per kit including NSB)

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Where:  
+R = Positive Reference Serum  
-R = Negative Reference Serum  
BLK = Blank wells (buffer in place of serum)  
U = Unknowns

REAGENT PREPARATION

1. **Positive Reference Serum**: Dilute 1:100 in EIA Phosphate Buffer, i.e., 20 µL serum + 1.98 mL EIA Buffer. For best results the solution should be used the same day as it is made.

2. **Negative Control Serum**: Dilute 1:100 in EIA Phosphate Buffer, i.e., 20 µL serum + 1.98 mL EIA Buffer. For best results the solution should be used the same day as it is made.

3. **Anti-Canine IgG-HRP**: Dilute 1:1000 in EIA Phosphate Buffer, i.e., 12 µL HRP + 12 mL EIA Buffer. For best results the solution should be used the same day as it is made.

4. **Wash Buffer**: Add 25 mL of 10x Wash Buffer concentrate to 225 mL of deionized water. Wash Buffer is stable until the expiration date when stored at 4°C.

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ASSAY PROCEDURE

WITHOUT NON-SPECIFIC BINDING SUBTRACTION (MBS480810 kit)
1. Mix all reagents thoroughly without foaming before use.
2. Determine the number of strips required to test the desired number of samples. Remove the remaining strips from the holder and store them at 4°C in the foil pouch provided.
3. Dilute the Samples 1:100 in EIA Phosphate Buffer, i.e., 20 µL serum + 1.98 mL EIA Buffer.
4. Pipette 100 µL of diluted Samples and Reference Sera into the thyroglobulin pre-coated wells and incubate the plate for 2 hours at room temperature. Shaking is not necessary.
5. Wash wells according to the following procedure:
   a. Remove the contents of each well by inversion of the plate.
   b. Tap out the remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of 1x Wash Buffer.
   d. Let stand for 2-3 minutes.
   e. Remove the contents of each well by inversion of plate into an appropriate disposal device.
   f. Repeat procedure two more times and proceed to step “g”.
   g. Tap out the remaining contents of the plate onto a lint free paper towel then proceed to step 6.
6. Add 100 µL of diluted Rabbit Anti-Canine IgG-HRP solution to each well and incubate the plate for 1 hour at room temperature.
7. Wash the plate 3x following the wash procedure from step 5.
8. Add 200 µL of TMB Substrate to each well and allow it to react for 10 minutes at room temperature.
9. Add 50 µL of 3N H₂SO₄ to each well to stop the reaction.
10. Zero the plate reader using the blank wells and read the plate at an absorbance of 450 nm.

WITH SUBTRACTION OF NON-SPECIFIC BINDING (MBS480810 and MBS480811 kits or MBS480812 kit)
1. Mix all reagents thoroughly without foaming before use.
2. Determine the number of strips with thyroglobulin pre-coated wells (Tg⁺) and non-specific binding (Tg⁻) strips required to test the desired number of samples. Remove the remaining strips from the holder and store them at 4°C in the foil pouch provided.
3. Dilute the Samples 1:100 in EIA Phosphate Buffer, i.e., 20 µL serum + 1.98 mL EIA Buffer.
4. Pipette 100 µL of diluted Samples and Reference Sera into the thyroglobulin pre-coated wells (Tg⁺) and the non-specific binding (Tg⁻) wells and incubate the plate for 2 hours at room temperature. Shaking is not necessary.
5. Wash wells according to the following procedure:
   a. Remove the contents of each well by inversion of the plate.
   b. Tap out the remaining contents of the plate onto a lint free paper towel.
   c. Add 300 µL of 1x Wash Buffer.
   d. Let stand for 2-3 minutes.
   e. Remove the contents of each well by inversion of plate into an appropriate disposal device.
   f. Repeat procedure two more times and proceed to step “g”.
   g. Tap out the remaining contents of the plate onto a lint free paper towel then proceed to step 6.
6. Add 100 µL of diluted Rabbit Anti-Canine IgG-HRP solution to each well and incubate the plate for 1 hour at room temperature.
7. Wash the plate 3x following the wash procedure from step 5.
8. Add 200 µL of TMB Substrate to each well and allow it to react for 10 minutes at room temperature.
9. Add 50 µL of 3N H₂SO₄ to each well to stop the reaction.
10. Zero the plate reader using the blank wells and read the plate at an absorbance of 450 nm.

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CALCULATION OF RESULTS

Three methods may be used to calculate the results of TgAA assays. **Method A** is the preferred method and should be used when the non-specific binding has been determined. **Method B** is suggested for screening dogs for TgAA. Dogs that are positive or inconclusive using Method B should be retested using non-specific binding and Method A. **Method C** is the traditional method that assesses TgAA levels relative to a negative control.

Recent research indicates that up to 10% of the dogs testing positive using Method C are false positives due to non-specific binding. It has been reported that recent immunizations can increase non-specific binding and thus false positives using Methods B or C. To eliminate this source of interference, we recommend that Method A be used for all dogs that test positive using Methods B or C. For dogs with inconclusive results, we recommend retesting dogs after three to six months.

A. Preferred Method

1. Record the absorbance at 450 nm for each well.
2. Average and record the values for the Positive and Negative Sera. Subtract the average of the corresponding non-specific binding wells from the Positive and Negative Sera.
3. Average and record the values for each Sample. Subtract the average of the corresponding non-specific binding wells from the average of each Sample.
4. Divide each Sample by the Positive Reference Serum and multiply by 100.
   - Samples over 25% are **Positive** for TgAA
   - Samples from 10-25% are **Inconclusive** for TgAA
   - Samples below 10% are **Negative** for TgAA

B. Screening Method (No NSB Subtraction)

1. Record the absorbance at 450 nm for each well.
2. Average and record the values for the Positive and Negative Sera. Subtract the average of the blank wells from the average of the Positive and Negative Sera.
3. Average and record the values for each Sample. Subtract the average of the blank wells from the average of each Sample.
4. Divide each Sample by the Positive Reference Serum and multiply by 100.
   - Samples over 35% are **Positive** for TgAA
   - Samples from 20-35% are **Inconclusive** for TgAA
   - Samples below 20% are **Negative** for TgAA

C. Traditional Method (No NSB Subtraction)

1. Record the absorbance at 450 nm for each well.
2. Average and record the values for the Positive and Negative Sera and for each sample.
3. Compute the standard deviation for the Positive and Negative Sera.
4. Compare the Sample averages with the Negative Reference Serum. Absorbance values less than two standard deviations greater than the Negative Reference Sera at a dilution of 1:100 are considered **Negative** for TgAA. Values greater than twice that of the Negative Reference Serum are considered **Positive** for TgAA. Intermediate values (more than two standard deviations above the Negative Reference Serum but less than twice that of the Negative Reference Serum) are considered **Inconclusive** and should be retested.

REFERENCES


*FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES*
8. Nachreiner, R.F., *et al.*; Canine TgAA assay modification to reduce false positive results from non-specific binding. Society of Comparative Endocrinology, 2003 annual meeting.