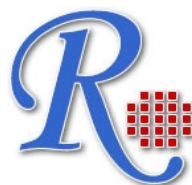


RayBio® Mouse DAN ELISA Kit

**User Manual
(Mar 25, 2013)**

**RayBio® Mouse DAN ELISA
Kit Protocol**

(Cat#: ELM-DAN-001)



RayBiotech, Inc.

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**RayBio® Mouse DAN
ELISA Kit Protocol**

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I. INTRODUCTION

The RayBio® Mouse DAN ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of Mouse DAN in serum, plasma and cell culture supernatants. This assay employs an antibody specific for DAN coated on a 96-well plate. Standards and samples are pipetted into the wells and DAN present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Mouse DAN antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of DAN bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. REAGENTS

1. DAN Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-Mouse DAN.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials, recombinant Mouse DAN.
4. Assay Diluent C (Item L): 30 ml of diluent buffer. For Standard/ Sample (serum/plasma samples/cell culture supernatants) diluent.
5. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. For detection antibody and HRP-Streptavidin diluent.
6. Detection Antibody DAN (Item F): 2 vial of biotinylated anti-Mouse DAN (each vial is enough to assay half microplate).
7. HRP-Streptavidin concentrate (Item G): 200 μ l 250x concentrated HRP-conjugated streptavidin.
8. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
9. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.

III. STORAGE

May be stored for up to 6 months at 2° to 8°C from the date of shipment. Standard (recombinant protein) should be stored at -20 °C or -80 °C (recommended at -80 °C) after reconstitution. Opened Microplate Wells or reagents may be stored for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20 °C. Avoid repeated freeze-thaw cycles.

IV. ADDITIONAL MATERIALS REQUIRED

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- 2 Precision pipettes to deliver 2 µl to 1 ml volumes.
- 3 Adjustable 1-25 ml pipettes for reagent preparation.
- 4 100 ml and 1 liter graduated cylinders.
- 5 Absorbent paper.
- 6 Distilled or deionized water.
- 7 Log-log graph paper or computer and software for ELISA data analysis.
- 8 Tubes to prepare standard or sample dilutions.

V. REAGENT PREPARATION

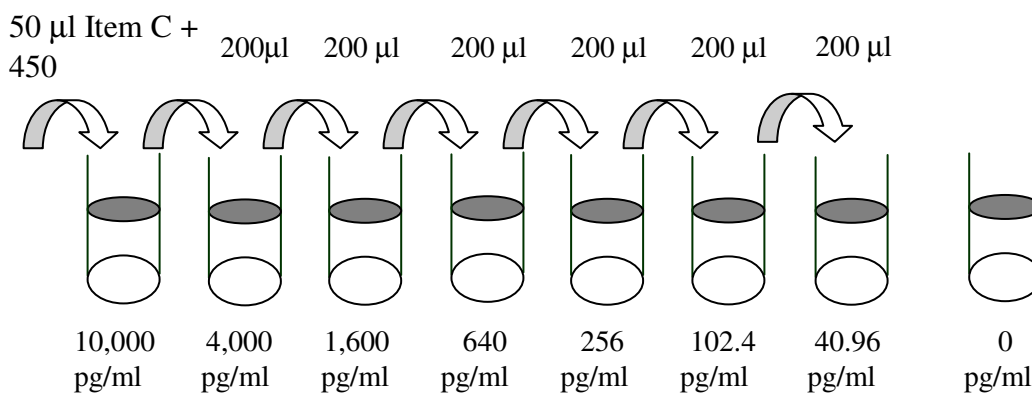
1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Sample dilution: If your samples need to be diluted, Assay Diluent C (Item L) should be used for dilution of serum/plasma/culture supernatants.

Suggested dilution for normal serum/plasma: 2 fold*.

* Please note that levels of the target protein may vary between

different specimens. Optimal dilution factors for each sample must be determined by the investigator.

3. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water before use.
4. Preparation of standard: **Briefly spin the vial of Item C.** Add 400 μl Assay Diluent C (Item L) into Item C vial to prepare a 50 ng/ml standard solution. **Dissolve the powder thoroughly by a gentle mix.** Add 50 μl DAN standard from the vial of Item C, into a tube with 450 μl Assay Diluent C to prepare a 10,000 pg/ml standard solution. Pipette 300 μl Assay Diluent C into each tube. Use the 10,000 pg/ml standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Assay Diluent C serves as the zero standard (0 pg/ml).



5. If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 μl of 1x Assay Diluent B (Item E) into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently

(the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent B and used in step 4 of Part VI Assay Procedure.

7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 250-fold with 1x Assay Diluent B (Item E).

For example: Briefly spin the vial (Item G) and pipette up and down to mix gently. Add 40 µl of HRP-Streptavidin concentrate into a tube with 10 ml 1x Assay Diluent B to prepare a 250-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.

VI. ASSAY PROCEDURE:

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 µl of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking.
3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 μ l of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 μ l of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. Add 100 μ l of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
9. Add 50 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.



2. Add 100 μ l standard or sample to each well.
Incubate 2.5 hours at room temperature or over night at 4°C.



3. Add 100 μ l prepared biotin antibody to each well.
Incubate 1 hour at room temperature.



4. Add 100 μ l prepared Streptavidin solution.
Incubate 45 minutes at room temperature.



5. Add 100 μ l TMB One-Step Substrate Reagent to each well.
Incubate 30 minutes at room temperature.



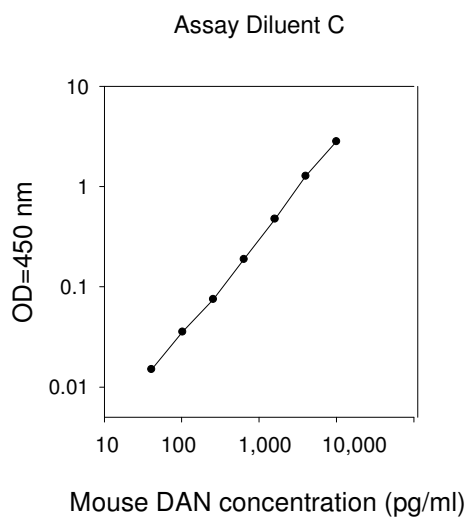
6. Add 50 μ l Stop Solution to each well.
Read at 450 nm immediately.

VIII. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable dose of DAN is typically less than 35 pg/ml.

C. RECOVERY

Recovery was determined by spiking various levels mouse DAN into mouse serum, plasma and cell culture media. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	84.40	74-94
Plasma	91.38	83-99
Cell culture media	108.1	78-121

D. LINEARITY

Sample Type		Serum	Plasma	Cell Culture Media
1:2	Average % of Expected	110.0	107.0	116.4
	Range (%)	102-118	99-115	103-130
1:4	Average % of Expected	128.3	120.3	120.4
	Range (%)	120-136	111-129	107-134

E. REPRODUCIBILITY

Intra-Assay: CV<10%

Inter-Assay: CV<12%

IX. SPECIFICITY

This ELISA pair antibody detects mouse DAN, no cross-reactivity with rhDAN, rhCOCO, rmBMP-4 or rmGremlin

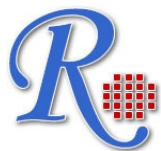
X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol style="list-style-type: none"> 1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution 	<ol style="list-style-type: none"> 1. Ensure sufficient incubation time; assay procedure step 2 change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	<ol style="list-style-type: none"> 1. Inaccurate pipetting 	<ol style="list-style-type: none"> 1. Check pipettes
4. High background	<ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash buffer 	<ol style="list-style-type: none"> 1. Review the manual for proper wash. If using an a plate washer, check that all ports are unobstructed. 2. Make fresh wash buffer
5. Low sensitivity	<ol style="list-style-type: none"> 1. Improper storage of the ELISA kit 2. Stop solution 	<ol style="list-style-type: none"> 1. Store your standard at $<-20^{\circ}\text{C}$ after reconstitution, others at 4°C. Keep substrate solution protected from light 2. Stop solution should be added to each well before measure

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