RayBio® Custom Binding Assay Kit

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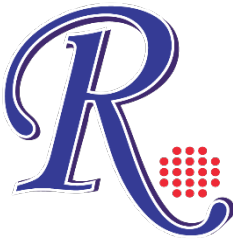
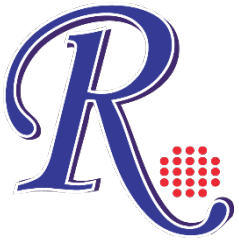


RayBiotech Life, Inc.

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



RayBiotech Life, Inc.

RayBio® Custom Binding Assay Kit Protocol

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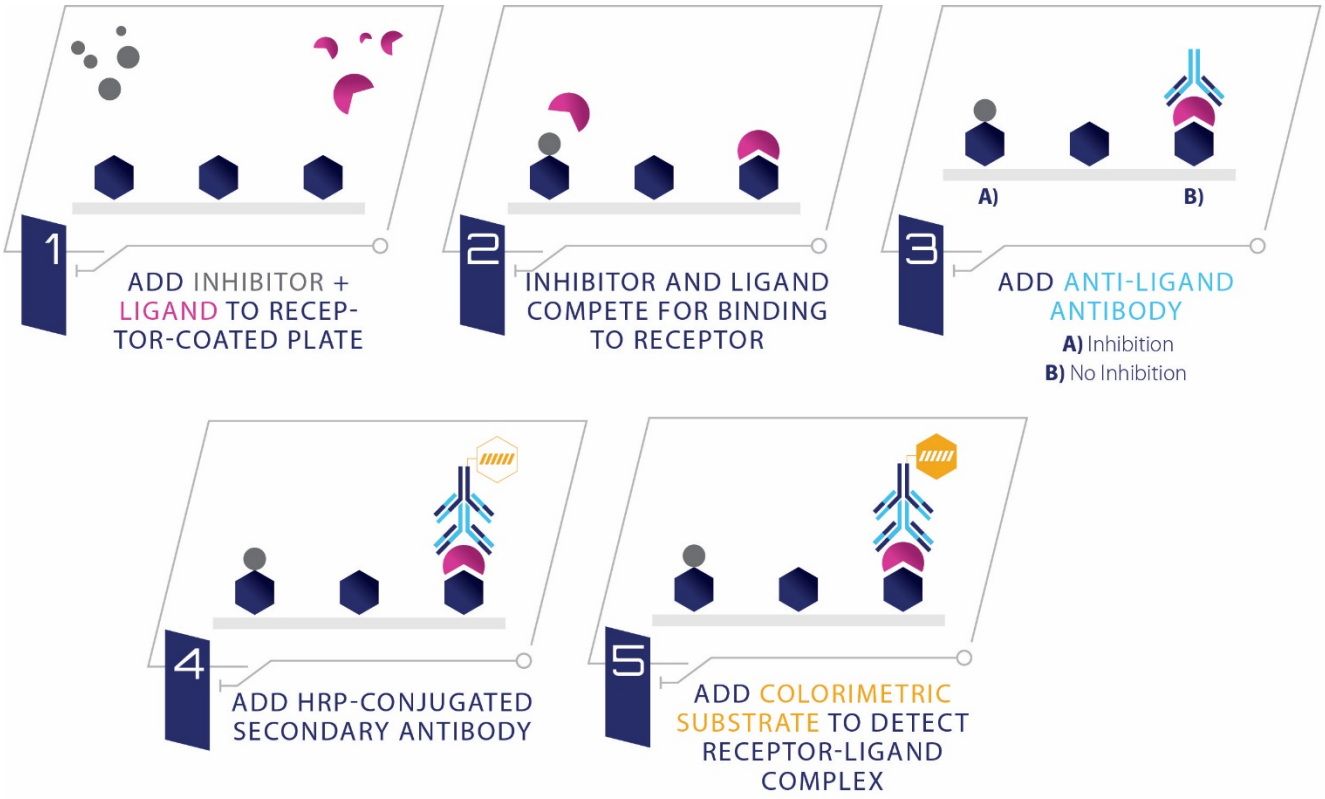
I. INTRODUCTION

The RayBio® custom binding assay kit is a patented, rapid, simple, and sensitive method to characterize the binding affinity between the target ligand and receptor proteins in the presence of potential inhibitors within 4 hours. The kit is compatible with various inhibitor types, including small molecules, peptides, antibodies, serum, or plasma, for neutralizing activity against the target ligand. Using this approach, inhibitors can be screened in a high throughput fashion, accelerating the development of therapeutic antibodies and drugs.

The RayBio® custom binding assay kit uses a 96-well plate coated with receptor protein. The testing reagent-of-choice is then added to the wells in the presence of target ligand. Unbound ligand is removed with washing, and a mouse anti-ligand detection antibody is added, binding to the ligand-receptor complex. After washing, an HRP-conjugated anti-secondary IgG is then applied to the wells in the presence of 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The HRP reacts with the TMB solution, producing a blue color that is proportional to the amount of bound ligand. The HRP-TMB reaction is halted with the addition of the Stop Solution, resulting in a blue-to-yellow color change. The intensity of the yellow color is then measured at 450 nm.



A schematic showing how the **RayBio Custom Binding Assay Kit** can measure the inhibition of the receptor-ligand interaction in the presence of a potential inhibitor.

II. STORAGE

The kit may be stored at 4°C up to 1 month from the date of shipment.

III. MATERIALS PROVIDED

COMPONENT	SIZE / DESCRIPTION	STORAGE / STABILITY AFTER PREPARATION
Receptor Protein-coated Microplate (Item A)	96 wells (12 strips x 8 wells) coated with receptor protein	1 month at 4°C*
Wash Buffer Concentrate (20x) (Item B)	25 ml of 20x concentrated solution	1 month at 4°C
5x Assay Diluent (Item E2)	15 ml of 5x concentrated buffer**	1 month at 4°C
Ligand Protein (Item F)	2 vials of ligand protein (1 vial is enough to assay half of the microplate)	5 days at 4°C
Detection Antibody Ligand Protein (Item C-1)	2 vials of anti-ligand antibody (1 vial is enough to assay half of the microplate)	5 days at 4°C
HRP-Conjugated Anti-IgG (Item D-2)	15 µl of 1000x concentrated HRP-Conjugated Anti-IgG	5 days at 4°C
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid	N/A

**Return unused wells to the pouch containing desiccant pack, reseal along entire edge.*

***This item is used to dilute the "Test Reagent" (i.e., the potential inhibitor), Ligand Protein (Item F), Detection Antibody Ligand Protein, and HRP-Conjugated Anti-IgG (Item D-2).*

IV. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Shaker.
3. Pipettes capable of accurately delivering 2 µl to 1 ml volumes.
4. Pipettes capable of delivering 1 – 25 ml volumes for reagent preparation.
5. Graduated cylinders: 100 ml and 1 liter
6. Distilled or deionized water.
7. Tubes to prepare sample dilutions.

V. REAGENT PREPARATION

1. Bring all kit reagents and “Test Reagents” to room temperature (18 - 25°C) before use.

   Note: *“Test Reagents” refers to the samples, or potential inhibitors, to be tested. The binding assay is compatible with various types of Test Reagents, including serum, plasma, peptides, antibodies, small molecules, and proteins.*

2. Dilute **5x Assay Diluent (Item E2)** 5-fold with deionized or distilled water before use to make “1x Assay Diluent.”

   Note: *A 5-fold dilution is the same as 1 part Assay Diluent and 4 parts water.*

3. If the **Wash Buffer Concentrate (20x) (Item B)** contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 ml of Wash Buffer Concentrate with 475 ml deionized or distilled water to yield 500 ml of “1x Wash Buffer.”

   Note: *500 ml of 1x Wash Buffer is enough to wash the plate as recommended.*

4. Briefly spin the **Ligand Protein (Item F)** before use. Add **50 µl** of 1x Assay Diluent into the **Ligand Protein** vial to prepare a “100x Ligand Protein Concentrate.” This protein concentrate will be used in Section VI, steps 3 and 4.

5. Dilute **50 µl** of the 100x Ligand Protein Concentrate prepared in the previous step 100-fold with 1x Assay Diluent to yield a “**1x Protein Solution**,” which will be used in Section VI, steps 5 and 6.

   Note: The volume of 100x Ligand Protein Concentrate listed here (50 µl) may not be appropriate for all experiments. The researcher should determine how much 100x Ligand Protein Concentrate should be diluted for their experiment prior to this step.

6. Briefly spin the **Detection Antibody Ligand Protein (Item C-1)** before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a 55x detection antibody concentrate. Pipette this detection antibody concentrate up and down to mix gently. This detection antibody concentrate should then be diluted 55-fold with 1x Assay Diluent to yield a “1x Detection Antibody” solution. Each reaction requires 100µl of 1x Detection Antibody solution, thus, the researcher should create enough volume to perform the desired number of reactions for each experiment.

   Note: In the assay protocol, 100 µl of 1x Detection Antibody will be eventually added per well. Scale up according to the number of wells utilized in the assay. Moreover, it is recommended that the calculated volume is multiplied by 1.2 to compensate for pipetting error and ensure there is enough volume to fill all wells.

7. Briefly spin the **HRP-Conjugated Anti-IgG (Item D-2)** before use. HRP-Conjugated Anti-IgG should be diluted 1000-fold with 1x Assay Diluent to yield a “1x HRP-conjugated IgG” solution. Each reaction requires 100µl of 1x HRP-conjugated IgG solution, thus, the

researcher should create enough volume to perform the desired number of reactions for each experiment.

Note: In the assay protocol, 100 µl of 1x HRP-Conjugated IgG will be eventually added per well. Scale up according to the number of wells utilized in the assay. Moreover, it is recommended that the calculated volume is multiplied by 1.2 to compensate for pipetting error and ensure there is enough volume to fill all wells.

VI. "TEST REAGENT" SAMPLE PREPARATION

General Considerations

Dose Response. When evaluating a potential inhibitor (i.e., the Test Reagent) of the ligand-receptor complex, it is highly recommended to perform a titration curve. This approach will help the researcher to empirically determine the dose-responsive range of Test Reagent as well as the lowest level that yields detectable inhibition. It will also help confirm whether the inhibition is real; that is, whether the inhibition increases as more Test Reagent is applied.

Note that the researcher should determine an appropriate serial dilution based on the known properties of their Test Reagent. From the result of this dilution series, the best dilution of the Test Reagent can be empirically determined.

Preparation of Reactions. Since the ligand protein putatively competes with the Test Reagent for binding to receptor, it is critical that *ligand be present at the same concentration in every well*. That is, the concentration of ligand protein is held constant while the Test Reagent varies from well to well.

Replicates. It is recommended that at least 2 replicates are run for all Test Reagents and controls. Therefore, replicates should be taken into account when calculating the volumes to be prepared. Each well will contain a final volume of 100 µL.

Serial Dilution Preparations.

1. Label a series of tubes, which will be used for the Test Reagent's serial dilutions.
2. *Optional (recommended):* Label a parallel set of tubes, which will be used for the Vehicle Blank's serial dilutions

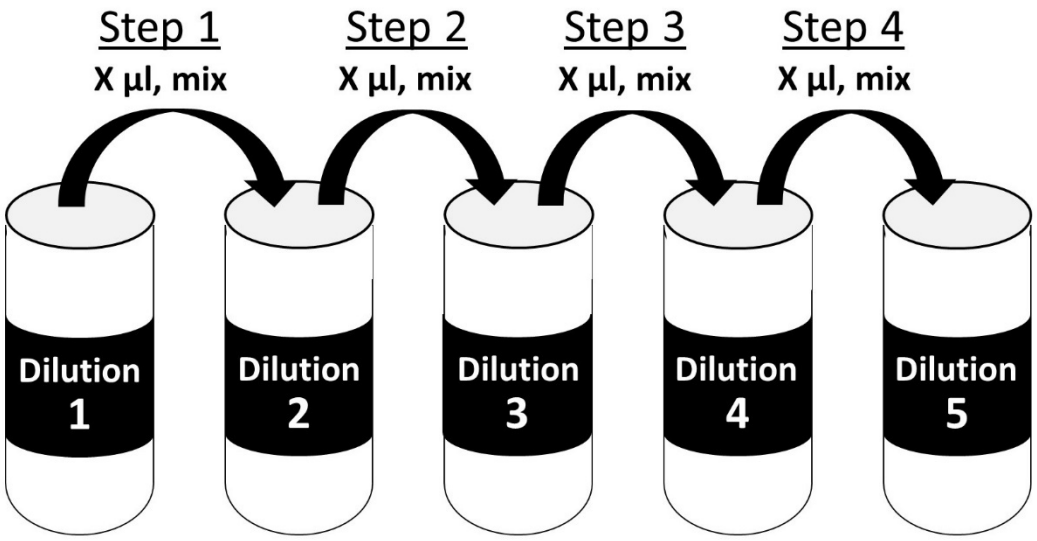
   Note: *The vehicle blank is the solution or buffer that the Test Reagent is in. For example, if the Test Reagent is in dimethyl sulfoxide (DMSO), a parallel set of tubes should be prepared with 50 µl of DMSO.*

3. To create the most concentrated dilution of the Test Reagent in the dilution series, mix the following together *per replicate*: Test Reagent + 1.25 µL 100x Ligand Protein Concentrate (prepared on page 5 in Reagent Preparation, Section V, step 4). Volume up to 125 µL with 1x Assay Diluent.

Note: *125 µL per replicate is noted here to account for pipetting errors and sample loss on the side of the tube. A final volume of 100 µL will be added per well.*

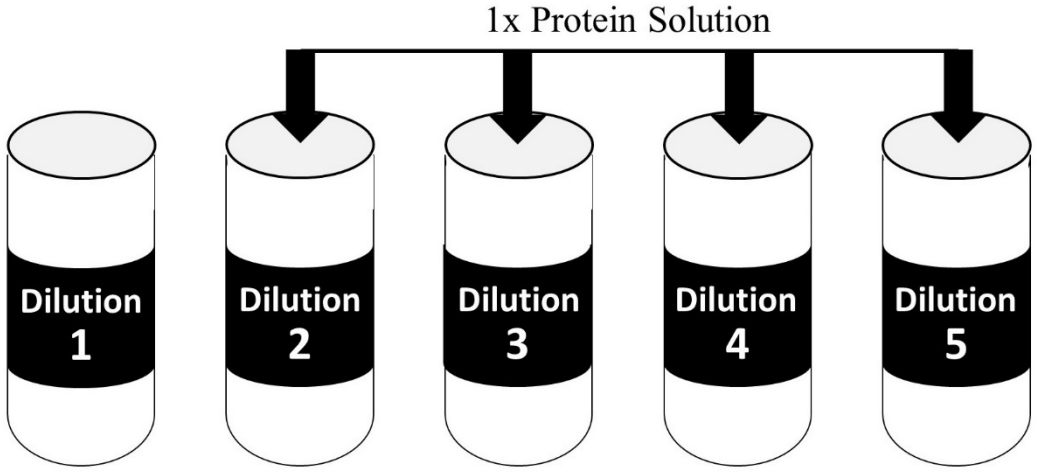
4. *Optional (recommended):* To create the most concentrated dilution of the Vehicle Blank in the dilution series, mix the following together *per replicate*: Vehicle Blank + 1.25 µL 100x Ligand Protein Concentrate (prepared on page 5 in Reagent Preparation, Section V, step 4). Volume up to 125 µL with 1x Assay Diluent.

5. Create remaining serial dilutions.
    a. Pipette X µL from the most concentrated dilution tube, “Dilution 1,” and place in the second most concentrated dilution tube, “Dilution 2.” Volume up to 125 µL with 1x Protein Solution (prepared on page 5 in Reagent Preparation, Section V, step 5) per replicate. Repeat for each serial dilution, using X µL of the prior concentration until the final concentration is reached.



Example: For a 1:10 serial dilution, pipette 12.5 µL of Dilution 1 into the second serial dilution tube, “Dilution 2,” containing 112.5 µL of 1x Protein Solution per replicate. Thoroughly mix and then repeat until all dilutions are prepared.

    b. Add **1x Protein Solution** (prepared on page 5 in Reagent Preparation, Section V, step 5), such that the final volume of dilutions is 125 µL per replicate.



Note: *The number of dilutions and the amount of dilution should be determined empirically by the researcher.*

6. Pipette 125 µl of the **1x Protein Solution** into a separate tube labeled “0 mM.” Mix thoroughly. This vial contains no Test Reagent and will serve as the **“0 mM” positive control**.

   NOTE: This positive control is necessary to determine whether the Test Reagent is an inhibitor. You will need to include this control sample every time this assay is performed. This control is required even after the optimum dilution is determined.

7. Pipette 125 µl of the **1x Assay Diluent** into a separate tube labeled “Reagent Blank.” This vial does *not* contain a Test Reagent or the 1x Protein Solution and will serve as the **“Reagent Blank” negative control**.

   NOTE: If you do not include the Vehicle Blank controls, you will need to include this Reagent Blank negative control sample every time this assay is performed to account for background noise. This control is required even after the optimum dilution is determined.

   NOTE: Use of Vehicle Blank controls are preferred over the Reagent Blank control.

VII. ASSAY PROCEDURE

1. Bring all reagents to room temperature (18 - 25°C) before use.

2. Label removable 8-well strips as appropriate for your experiment.

3. As prepared above in Section VI, **add 100 µl of each Test Reagent** into an appropriate well.

4. Cover wells with the provided plate sealing film and incubate for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.

5. Discard the solution and **wash 4 times** with 1x Wash Solution. Wash by filling each well with 1x Wash Buffer (300 µl) using a multi-channel pipette or auto-washer. 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.

6. Add 100 µl of prepared **1x Detection Antibody** (see Reagent Preparation, Section V, Step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.

7. Discard the solution. Repeat the wash as described in Step 5 above.

8. Add 100 µl of the prepared **1x HRP-Conjugated Anti-IgG** (see Reagent Preparation, Section V, Step 7) to each well. Incubate for 1 hour at room temperature with gentle shaking.

9. Discard the solution. Repeat the wash as described in Step 5 above.

10. Add 100 µl of **TMB One-Step Substrate Reagent** (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

11. Add 50 µl of **Stop Solution** (Item I) to each well.

12. Read at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents and Test Reagent samples as instructed.

2. Add 100 µl Test Reagent samples to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.

3. Add 100 µl the prepared 1X Detection antibody solution to each well. Incubate 1 hour at room temperature.

4. Add 100 µl the prepared 1X HRP-conjugated IgG antibody solution to each well. Incubate 1 hour at room temperature.

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

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

IX. DATA ANALYSIS

1. Determine the average optical density (OD) absorbance across the replicate readings for each Test Reagent and control. *These average ODs will be used for all further calculations.*
2. Subtract the “Vehicle Blank” or “Reagent Blank” OD values from your Test Reagent OD values.
3. Compare the Test Reagent data to the “0 mM” positive control data where no Test Reagent was added.

   Note: *A Test Reagent’s sample absorbance will be lower than the positive control if the ligand-receptor interaction is inhibited.*

4. Determine the percent binding inhibition (BI%):

   BI% = [(OD of “0 mM” positive control – OD of Test Reagent) / OD of “0 mM” positive control)] x 100

X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Too high sample signal	Sample concentration is too low	Increase sample concentration
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low "no sample" signal	Improper storage of kit	Upon receipt, the kit should be stored at 4°C
	Stop Solution	Stop Solution should be added to each well. The OD should be read immediately after adding the Stop Solution.
	Improper dilution of protein, primary or secondary antibody	Ensure correct dilution