CD47: SIRP alpha [Biotinylated] Inhibitor Screening ELISA Assay Pair

Pack Size: 96 tests / 480 tests

Catalog Number： EP-102

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use In Diagnostic Or Therapeutic Procedures

PRINCIPLE OF THE ASSAY

Immune checkpoint pathway is a focal point of today's cancer research. CD47 is one of the best characterized checkpoint proteins. CD47 binding to SIRP alpha aids in tumor evasion of the immune system. Therefore, the pharmaceutical inhibition of CD47 has been considered a promising strategy by many oncologists.

This inhibitor screening ELISA pair is designed to facilitate the identification and characterization of new CD47 pathway inhibitors. This assay employs a simple colorimetric ELISA platform, which measures the binding between immobilized **human CD47** and in-house developed **biotinylated SIRP alpha** protein. This product is uniquely suitable for rapid high-throughput screening of putative CD47 inhibitors. Briefly, we provide you with a **biotinylated human SIRP alpha** protein, a **human CD47** protein, an **anti-CD47 neutralizing antibody** (*as method verified Reference*), and **Streptavidin-HRP** reagent. Your experiment will include 4 simple steps:

a) Coat the plate with **human CD47**.

b) Mix **biotinylated human SIRP alpha** and **your molecule of interest**.

c) Add to the coated **human CD47**.

d) Add **Streptavidin-HRP** followed by TMB or other colorimetric HRP substrate.

Finally, the ability of your compound to inhibit CD47: SIRP alpha binding will be determined by comparing OD readings among different experimental groups.

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MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED

Catalog	Components	Size (96tests)	Size (480tests)	Format	Storage
A004-214	Human CD47	35 μg	160 μg	Powder	-20℃
A005-214	Biotinylated Human SIRP alpha	10 μg	10 μg	Powder	-20℃
A003-214	Streptavidin-HRP	10 μg	10 μg	Powder	-20℃, avoid light
CD7-NA002	Anti-CD47 Neutralizing Antibody	20 μg	100 μg	Powder	-20℃

RECONSTITUTION

Reconstitute the provided lyophilized materials to stock solutions with PBS as recommended in **Table 2.1 and Table 2.2,** Solubilize for 15 to 30 minutes at room temperature with occasional gentle mixing. **Avoid vigorous shaking or vortexing**.

The reconstituted stock solutions should be stored at -70℃. **It is recommended not to freeze thaw more than 3 times.**

To avoid surface adsorption loss and inactivation, the reconstituted protein must NOT be aliquoted to less than 5 μg per vial.

***Note**: Streptavidin-HRP stock solution should be protected from light.*

TABLE 2.1. RECONSTITUTION METHODS FOR 96 TESTS

Catalog	Components	Size	Stock Solution Con.	Reconstitution Buffer and Vol.
A004-214	Human CD47	35 μg	250 μg/mL	140 μL PBS
A005-214	Biotinylated Human SIRP alpha	10 μg	100 μg/mL	100 μL PBS
A003-214	Streptavidin-HRP	10 μg	50 μg/mL	200 μL PBS
CD7-NA002	Anti-CD47 Neutralizing Antibody	20 μg	250 μg/mL	80 μL PBS

TABLE 2.2. RECONSTITUTION METHODS FOR 480 TESTS

Catalog	Components	Size	Stock Solution Con.	Reconstitution Buffer and Vol.
A004-214	Human CD47	160 μg	250 μg/mL	640 μL PBS
A005-214	Biotinylated Human SIRP alpha	10 μg	100 μg/mL	100 μL PBS
A003-214	Streptavidin-HRP	10 μg	50 μg/mL	200 μL PBS
CD7-NA002	Anti-CD47 Neutralizing Antibody	100 μg	250 μg/mL	400 μL PBS

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SHIPPING AND STORAGE

All components are shipped in lyophilized state at room temperature. This product is stable after storage at:

1) Room temperature (RT) for 1 month in lyophilized state.
2) -20℃ for 1 year in lyophilized state.
3) -70℃ for 6 months under sterile conditions after reconstitution.

MATERIALS OR INSTRUMENTS REQUIRED BUT NOT SUPPLIED (for 96 tests)

Coating Buffer PBS (Phosphate Buffered Saline), pH7.4, 12 mL is sufficient for 96 tests.

Wash Buffer PBS with 0.05% (v/v) Tween-20, 500 mL is sufficient for 96 tests.

Blocking Buffer Wash Buffer with 2% (w/v) bovine serum albumin (BSA) *(Sigma-Aldrich, Catalog # A4737),* 35 mL is sufficient for 96 tests.

Dilution Buffer Wash Buffer with 0.5% (w/v) bovine serum albumin (BSA) *(Sigma-Aldrich, Catalog # A4737),* 50 mL is sufficient for 96 tests.

Substrate Stock Solution 10 mg/mL TMB *(Sigma-Aldrich, Catalog # 860336)* in Dimethyl sulfoxide *(Sigma-Aldrich, Catalog # D8418)*, 1 mL is sufficient for 96 tests. **Protect from light**.

Substrate Dilution Buffer 50 mM disodium hydrogen phosphate (Na_2HPO_4) and 25 mM citric acid, adjust pH to 5.5 with 1 M Sodium hydroxide (NaOH), 25 mL is sufficient for 96 tests.

TMB Substrate Working Solution

For **each plate** dilute 250 µL substrate stock solution in 25 mL substrate dilution buffer and add 12 µL 5% H_2O_2 (pipette 10 µL 30% H_2O_2 into 50 µL distilled water), mix well.

Notes:

1) *The TMB Substrate Working Solution should be freshly prepared and used within 15 minutes.*
2) *If you choose to use other commercially available ready-to-use TMB substrate solutions, you should follow the manufacturer's instruction.*

Stop Solution 1 M sulfuric acid (aqueous), 6 mL is sufficient for 96 tests.

High binding surface 96-well microplate, clear flat bottom *(Corning, Catalog # 9018)*

Microplate sealing film *(Sigma-Aldrich, Catalog # Z724742)*

Pipettes and pipette tips

UV/Vis microplate spectrophotometer (absorbance 450nm, correction wavelength set to 630 nm).

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RECOMMENDED PROTOCOL

1. **Preparation**

   Reconstitute and store all reagents as recommended.

2. **Coating**

   1) Dilute **human CD47** stock solution (250 μg/mL) to 3 μg/mL with **Coating Buffer** to make **human CD47** working solution.
   2) Please leave two wells uncoated for **No-Coating Control (Table 3)**.
   3) Add 100 μL of **human CD47** working solution (3 μg/mL) to each well, seal the plate with microplate sealing film and incubate overnight (or 16 hours) at 4℃.

3. **Washing**

   Remove the remaining solution by aspiration, add 300 μL of **Wash buffer** to each well, gently tap the plate for 1 minute, remove any remaining **Wash Buffer** by aspirating or decanting, invert the plate and blot it against paper towels. **Repeat the wash step above for three times**.

   Note: *For best results, the complete removal of the* ***human CD47*** *solution is essential. The use of a manifold dispenser or an auto-washer may be necessary.*

4. **Blocking**

   Add 300 μL **Blocking Buffer** to each well, seal the plate with microplate sealing film and incubate at 37℃ for 1.5 hours.

5. **Washing**

   Repeat step 3. At meantime, you can start to prepare your **samples**.

6. **Add Samples**

   1) Dilute **biotinylated human SIRP alpha** stock solution (100 μg/mL) to 0.25 μg/mL with **Dilution Buffer** to make **biotinylated human SIRP alpha** working solution.
   2) Make series dilution of the samples as appropriate, then mixed with same volume **biotinylated human SIRP alpha** working solution (For example: 110 μL **biotinylated human SIRP alpha** working solution + 110 μL diluted samples).
   3) If you intend to use the provided **anti-CD47 neutralizing antibody** as a reference (Ref.), you may dilute the antibody as recommended in **Figure 1**, then mixed with same volume **biotinylated human SIRP alpha** working solution (For example: 110 μL **biotinylated human SIRP alpha** working solution + 110 μL diluted **anti-CD47 neutralizing antibody**).
   4) For **No-Coating Control** wells, please mix 110 μL **Dilution Buffer** and 110 μL **Sample Buffer**.
   5) For **No-Binding Control** wells, please mix 110 μL **Dilution Buffer** and 110 μL **Sample Buffer**.
   6) For **Positive Control** wells, please mix 110 μL **biotinylated human SIRP alpha** working solution and 110 μL **Dilution Buffer**.
   7) Add 100 μL mixer to the wells according to our recommendation (**Figure 2**) or your own plate setup. Seal the plate with microplate sealing film and incubate at 37℃ for 1 hour.

   Note: The working solution should be prepared immediately before use and should not be stored.

7. **Washing**

   Repeat step 3.

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8. Streptavidin-HRP Labeling

1) Dilute **Streptavidin-HRP** stock solution (50 μg/mL) to 0.1 μg/mL with **Dilution Buffer** to make **Streptavidin-HRP** working solution.
2) For all wells, add 100 μL **Streptavidin-HRP** working solution, seal the plate with microplate sealing film and incubate at 37℃ for 1 hour, **avoid light**.

9. Washing

Repeat step 3.

10. TMB Substrate Reaction

Add 200 μL **TMB Substrate Working Solution** to each well. Seal the plate with microplate sealing film and incubate at 37℃ for 20 minutes, **avoid light**.

11. Termination

Add 50 μL **Stop Solution** to each well, and tap the plate gently for 3 minutes to allow thorough mixing.

Note: *the color in the wells should change from blue to yellow.*

12. Data Recording

Read the absorbance at 450 nm using UV/Vis microplate spectrophotometer.

Note*: the plate may be read at 600 nm without adding 1 M sulfuric acid, but the Signal-to-Background ratio may be reduced.*

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FIGURE 1. PREPARATION OF 1:2 SERIAL DILUTIONS OF THE ANTI-CD47 NEUTRALIZING ANTIBODY

Tubes/ Solution Code	Anti-CD47 Neutralizing-Antibody stock solution	Ref.-1	Ref.-2	Ref.-3	Ref.-4	Ref.-5	Ref.-6	Ref.-7	Ref.-8
Operating	*20 µL*	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	
Solution Con.	250 µg/mL	10 µg/mL	5 µg/mL	2.5 µg/mL	1.25 µg/mL	0.625 µg/mL	0.313 µg/mL	0.156 µg/mL	0.078 µg/mL
Dilution Buffer Vol.		*480 µL*	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL	150 µL

FIGURE 2. PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ref.-8	Ref.-8	Positive Ctrl.	Positive Ctrl.	...	...	...	...	...	...	...	...
B	Ref.-7	Ref.-7	No-binding Ctrl.	No-binding Ctrl.	...	...	...	...	...	...	...	...
C	Ref.-6	Ref.-6	No-coating Ctrl.	No-coating Ctrl.	...	...	...	...	...	...	...	...
D	Ref.-5	Ref.-5	...	...	...	...	...	...	...	...	...	...
E	Ref.-4	Ref.-4	...	...	...	...	...	...	...	...	...	...
F	Ref.-3	Ref.-3	...	...	...	...	...	...	...	...	...	...
G	Ref.-2	Ref.-2	...	...	...	...	...	...	...	...	...	...
H	Ref.-1	Ref.-1	...	...	...	...	...	...	...	...	...	...

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TABLE 3. ASSAY PROTOCOL

Steps Code	Steps	Reagents & Instruments	Reaction Conditions	Samples	No-binding Control	No-coating Control	Positive Control
1	Preparation	N/A	N/A	N/A	N/A	N/A	N/A
2	Coating	Human CD47 Working Solution	4℃ for overnight	100 µL	100 µL	—	100 µL
3	Washing	Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
4	Blocking	Blocking Buffer	37℃ for 1.5 hours	300 µL	300 µL	300 µL	300 µL
5	Washing	Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
6	Add Samples	Biotinylated Human SIRP alpha Working Solution	Incubate at 37℃ for 1 hour	50 µL	—	—	50 µL
		Dilution Buffer		—	50 µL	50 µL	50 µL
		Samples		50 µL	—	—	—
		Sample Buffer		—	50 µL	50 µL	—
7	Washing	Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
8	Streptavidin-HRP Labeling	Streptavidin-HRP Working Solution	37℃ for 1 hours	100 µL	100 µL	100 µL	100 µL
9	Washing	Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
10	TMB Substrate Reaction	TMB Substrate Working Solution	37℃ for 20 minutes	200 µL	200 µL	200 µL	200 µL
11	Stop the Reaction	Stop Solution	Mix by gentle tapping for 3 minutes	50 µL	50 µL	50 µL	50 µL
12	Data Recording	UV/Vis spectrophotometer	Measure absorbance at 450 nm, with the correction wavelength set at 600 nm				

Note for TABLE 3:

1) ***Samples:*** *Your samples of interest.*
2) ***No-Binding Control:*** *Reaction without* ***biotinylated human SIRP alpha*** *added. The absorbance should be around 0.05(< 0.1) at 450 nm.*
3) ***No-Coating Control:*** *Reaction without* ***human CD47*** *coated on the wells. The absorbance should be around 0.05(< 0.1) at 450 nm.*
4) ***Positive Control:*** *Determined the max value in 450 nm absorbance, when out of inhibitors.*
5) *It is recommended that all samples, controls and references should be done in duplicates.*

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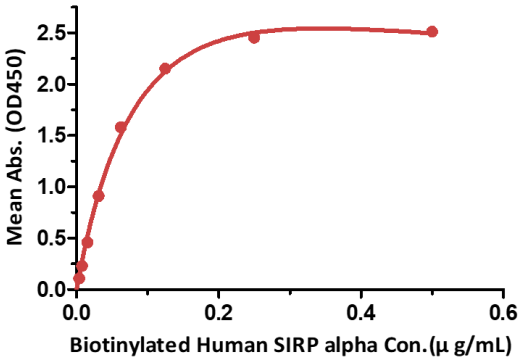
METHOD VERIFICATION

- **CD47: SIRP ALPHA [BIOTINYLATED] BINDING IN THE ABSENCE OF INHIBITORS**

Immobilized **human CD47** protein at 3 μg/mL (100 μL/well) can bind **biotinylated human SIRP alpha** with a linear range of 0.004-0.125 μg/mL when detected by **Streptavidin-HRP**. Background was subtracted from data points before curve fitting.

FIGURE 3. BINDING OF BIOTINYLATED HUMAN SIRP ALPHA TO IMMOBILIZED HUMAN CD47 IN A FUNCTIONAL ELISA ASSAY

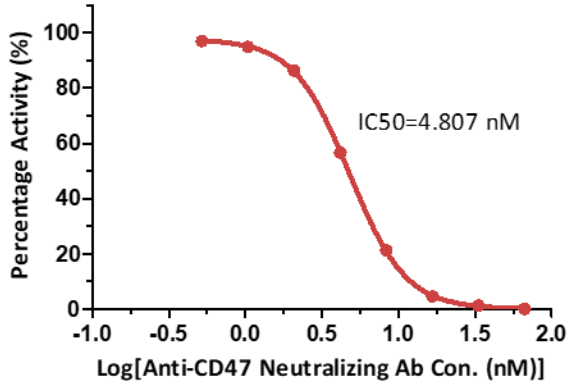


Biotinylated Human SIRP alpha (μg/mL)	Mean Abs.(OD450)
0.500	2.51
0.250	2.45
0.125	2.15
0.063	1.58
0.031	0.91
0.015	0.46
0.008	0.23
0.004	0.11

- **INHIBITION OF CD47: SIRP ALPHA [BIOTINYLATED] BINDING BY ANTI-CD47 NEUTRALIZING ANTIBODY**

Serial dilutions of anti-CD47 neutralizing antibody (Catalog # CD7-NA002) (1:2 serial dilutions, from 10 μg/mL to 0.04 μg/mL) was added into CD47: biotinylated SIRP alpha binding reactions. The assay was performed according to the above described protocol. Background was subtracted from data points prior to log transformation and curve fitting.

FIGURE 4. INHIBITION OF CD47: SIRP ALPHA [BIOTINYLATED] BINDING BY ANTI-CD47 NEUTRALIZING ANTIBODY



Anti-CD47 Neutralizing Ab Con. (μg/mL)	Anti-CD47 Neutralizing Ab Con. (nM)	Log[Anti-CD47 Neutralizing Ab Con. (nM)]	Mean Abs. (OD450)	Percent Activity (%)
0	0		2.59	100
0.036	0.261	-0.58	2.55	98.49
0.078	0.521	-0.28	2.51	97.03
0.156	1.042	0.02	2.46	94.94
0.313	2.083	0.32	2.24	86.41
0.625	4.167	0.62	1.46	56.56
1.25	8.333	0.92	0.55	21.22
2.5	16.667	1.22	0.12	4.54
5	33.333	1.52	0.03	1.26
10	66.667	1.82	0	0.06

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TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solutions
Signal of positive control is weak or abnormal	**Reconstituted protein be aliquoted to less than 5 μg per vial**	✧ **Reconstituted protein must NOT be aliquoted to less than 5 μg per vial.**
	The working solution not be prepared immediately before use	✧ **The working solution should be prepared immediately before use and should not be stored.**
	Biotinylated human SIRP alpha, **human CD47,** or **Streptavidin - HRP** may have lost activity	✧ Make sure your proteins are aliquoted into single-use aliquots. ✧ Increase the time of reaction or increase the protein concentration may help in case the protein activity is decreased over time.
	Errors in instrument settings	✧ Please check instrument setting.
	Substrate Stock Solution is outdated; Incubation temperature is incorrect; Incubation time is not sufficient; Repeated freeze-thaw cycles;	✧ Make sure the **Substrate Stock Solution** is working. ✧ Use proper incubation time and temperature.
	Pipetting errors	✧ Make sure that the pipette is calibrated and working properly.
High background	Insufficient washing or blocking	✧ Be sure the blocking step is performed. ✧ Increase number of washes and the volume **Wash Buffer** used. ✧ Increase Tween-20 concentration to 0.1% in **Wash Buffer**. ✧ Make sure **Streptavidin-HRP** is diluted in **Blocking Buffer**.
	Sample solvent contains inhibiting factors	✧ Run a negative control assay with the solvent alone. ✧ Maintain DMSO level at <1%. Increase protein incubation time.
	Contamination	✧ Make sure buffers and samples are prepared, used and stored correctly.
	The **TMB Substrate Working Solution** is not fresh	✧ **TMB Substrate Working Solution** must be used within 15 minutes after preparation.
Colorimetric signal is erratic	Inconsistent pipetting or dilution methods	✧ Make sure pipettors are functioning properly and use a multichannel pipettor if possible. ✧ Use master mixes to minimize errors. ✧ Run duplicates for all tests.
	TMB Substrate Working Solution is not completely mixed with the reaction solution	✧ Make sure that **TMB Substrate Working Solution** is adequately mixed with the reaction solution.
	Bubbles in the wells	✧ Tap plate gently to disperse bubbles.
	Signal is too high	✧ The concentration of the samples should be adjusted to achieve optimal reading. ✧ Decrease colorimetric HRP substrate incubation time.
Inadequate color development	Incomplete removal of residual buffers during previous steps	✧ Wells should appear dry after aspiration.
	Problems with conjugate or color reagents	✧ Color should appear immediately after the reagent is added. Make sure no contamination or residual buffers in the wells before you start the color development process.

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