ELISA Assay Kit for Anti-CTLA-4 h-mAb in Monkey Serum

Pack Size: 96 tests / 480 tests

Catalog Number: ECC-V1

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

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

Intended Use

The enclosed kit is intended for research use only. The products are not intended for treatment and/or diagnosis of diseases.

Background

How a therapeutic antibody is metabolized in the body is pertinently relevant to its efficacy. Therefore, pharmacokinetics study is an important part of the drug development.

CTLA-4 is one of the most targeted molecules in today's pharmaceutical industry, thanking to the clinical success of Yervoy®. Many investigational anti-CTLA-4 mAbs are being developed. There is a growing need for a standard assay that can be used to facilitate the study of their pharmacokinetics.

Assay Principles

The enclosed ELISA assay kit for anti-CTLA-4 h-mAb in monkey serum is based on an enzyme immunoassay(ELISA) between recombinant CTLA-4 protein (ECD) and the biotinylated anti-CTLA-4 monoclonal antibody. The method employs the principle of competitive ELISA, allowing quantification of the monoclonal anti-CTLA-4 antibodies in monkey serum.

The assay involves the following steps:

a) Coat the plate with human CTLA-4;

b) Add the mixture of your sample and the biotinylated anti-CTLA-4 antibody;

c) Add Streptavidin-HRP followed by TMB or other colorimetric HRP substrate;

d) Record the OD readings and analyze the serum concentration.

Advantages

- **Wide coverage**

  There is no requirement for the species and subtype of the antibody.

- **Easy process**

  There is no longer a need to generate anti-drug antibodies for PK studies.

- **Clean result**

  The use of the biotinylated antibody in the competitive ELISA significantly alleviates the issue of background noise issue that often occurs in traditional ELISA method.

Materials Provided

Tab.1 Materials Provided

Catalog	Components	Size (96 tests)	Size (480 tests)	Format	Storage
PK901-213	Human CTLA-4	15 μg	60 μg	Powder	-20℃
PK902-213	Biotinylated anti-CTLA-4 antibody	10 μg	10 μg	Powder	-20℃
PK903-213	Streptavidin-HRP	5 μg	10 μg	Powder	-20℃, avoid light
PK904-213	Anti-CTLA-4 antibody	30 μg	30 μg	Powder	-20℃

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Reconstitution

Reconstitute the provided lyophilized materials to stock solutions with PBS as recommended in **Tab.2.1 and Tab 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 -80℃. **Avoid freeze-thaw cycles.**

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

Tab. 2.1 Reconstitution Method for 96 Tests

Catalog	Components	Size	Stock Solution Con.	Reconstitution Buffer and Vol.
PK901-213	Human CTLA-4	15 μg	250 μg/mL	60 μL PBS
PK902-213	Biotinylated anti-CTLA-4 antibody	10 μg	50 μg/mL	200 μL PBS
PK903-213	Streptavidin-HRP	5 μg	50 μg/mL	100 μL PBS
PK904-213	Anti-CTLA-4 antibody	30 μg	200 μg/mL	150 μL PBS

Tab. 2.2 Reconstitution Method for 480 Tests

Catalog	Components	Size	Stock Solution Con.	Reconstitution Buffer and Vol.
PK901-213	Human CTLA-4	60 μg	250 μg/mL	240 μL PBS
PK902-213	Biotinylated anti-CTLA-4 antibody	10 μg	50 μg/mL	200 μL PBS
PK903-213	Streptavidin-HRP	10 μg	50 μg/mL	200 μL PBS
PK904-213	Anti-CTLA-4 antibody	30 μg	200 μg/mL	150 μL PBS

Minimal Volume for Aliquot(s)

To avoid surface adsorption loss and inactivation, the reconstituted protein must NOT be aliquotedto less than 10 μg per vial.

Shipping and Storage

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

1) Room temperature (RT) for 1 month in lyophilized state;
2) -20℃ for 6 months in lyophilized state;
3) -80℃ for 4 months under sterile conditions after reconstitution.

Materials or Instruments Required But Not Supplied

Coating Buffer PBS (Phosphate Buffered Saline), pH7.4, 12 mL is sufficient.
Wash Buffer PBS with 0.05% (v/v) Tween-20 (PBST), 500 mL is sufficient.
Blocking Buffer Wash Buffer with 2%(w/v) bovine serum albumin (Sigma-Aldrich,Catalog # A4737),35 mL is sufficient.
Dilution Buffer Wash Buffer with 0.5% (w/v)bovine serum albumin(Sigma-Aldrich,Catalog # A4737),50 mL is sufficient.

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Substrate Stock Solution
10 mg/mL TMB (Sigma-Aldrich, Catalog # 860336) in dimethyl sulfoxide (Sigma-Aldrich, Catalog # D8418), 1 mL is sufficient. **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.

TMB Substrate Working Solution
Dilute 25 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.
Therapeutic Anti-CTLA-4 Antibody to be Analyzed prepare by customer.
Serum Samples for Analyzed prepare by customer.
Monkey Serum Pre-dosed therapeutic serum or pooled by the equal amount of male and female serum as dilution matrix.
High Binding Surface 96-well Microplate, Clear Flat Bottom (Corning, Catalog # 42592)
Microplate Sealing Film (Sigma-Aldrich, Catalog # 28416050)
Pipettes and Pipette Tips
UV/Vis Microplate Spectrophotometer (Absorbance 450 nm, correction wavelength set to 600 nm)

Notes:
Pilot experiment may be required to estimate the range of antibody concentration in samples.

Recommended Protocol

1. Preparation
1.1. Reconstitute and store all reagents as recommended.
1.2. Use the **Therapeutic Anti-CTLA-4 Antibody** to make **5×Standards(STDs) Stock Solution** with monkey serum. The **5×STDs Stock Soltiuon** should include 100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.125 µg/mL, 1.5625 µg/mL, 0.78125 µg/mL, 0.390625 µg/mL ,0.1953125 µg/mL and 0 µg/mL of STDs respectively and stored frozen in single-use aliquots at -80°C until use.
1.3. Use the **Therapeutic Anti-CTLA-4 Antibody** to make **5×Quality Controls(QCs) Stock Solution** with monkey serum. The **5×QCs Stock Solution** should cover the quantification range of the standard curve (see below). Make independent dilutions from the antibody stock solution (e.g. include 25 µg/mL, 20 µg/mL, 5 µg/mL, 2µg/mL and 0.78525 µg/mL of QCs) and stored frozen in single-use aliquots at -80°C until use.
1.4. Use the provided **Anti-CTLA-4 Antibody** as a reference if you need; dilute the antibody with monkey serum as recommended in 1.2.

2. Coating
2.1. Dilute human **CTLA-4 Stock Solution** (250 µg/mL) to 1 µg/mL with **Coating Buffer** to make human **CTLA-4 Working Solution.**
2.2. Please leave a couple of wells uncoated for **STD-1 (No-Coating)** and **Only 10% Serum (No-Coating),** respectively **(Fig.1).**
2.3. Add 100 µL of human **CTLA-4 Working Solution** (1 µg/mL) to each well, seal the plate with microplate sealing film and incubate overnight (or 16 hours) at 4℃.

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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.**

4. Blocking

Add 300 µL **Blocking Buffer** to each well at 37℃ for 1.5 hours.

5. Washing

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

6. Add Samples

6.1. Dilute the **Biotinylated Anti-CTLA-4 Antibody Stock Solution** (50 µg/mL) to 0.02 µg/mL with **Dilution Buffer** to make **Biotinylated Anti-CTLA-4 Antibody Working Solution.**

6.2. Dilute **5×STDs** and **5×QCs** with **Dilution Buffer,** then mixed with same volume **Biotinylated Anti-CTLA-4 Antibody Working Solution.** (For example: 22 µL 5×STDs + 88 µL **Dilution Buffer** +110 µL **Biotinylated Anti-CTLA-4 Antibody Working Solution**).

6.3. Dilute patient **Samples** with same volume **Biotinylated Anti-CTLA-4 Antibody Working Solution.**

6.4. For **STD-1 (No-Coating),** dilute **5×STD-1** (100 µg/mL) with **Dilution Buffer,** then mixed with same volume **Biotinylated Anti-CTLA-4 Antibody Working Solution** (For example: 22 µL 5×STD-1 + 88 µL **Dilution Buffer** +110 µL **Biotinylated Anti-CTLA-4 Antibody Working Solution**). Add 100 µL mixer to the wells.

6.5. For **Only 10% Serum (No-Coating)** and **Only 10% Serum (Coating),** please add 100 µL **Dilution Buffer** with **10% Monkey Serum** (Fig. 1).

6.6. For **Only Dilution Buffer (Coating),** please add 100 µL **Dilution Buffer** (Fig. 1).

6.7. For all other wells, add 100 µL mixed STDs, QCs and Samples respectively, seal the plate with microplate sealing film and incubate at 37℃ for 1 hour.

7. Washing

Repeat step 3.

8. Streptavidin-HRP Conjugating

8.1. Dilute **Streptavidin-HRP Stock Solution** (50 µg/mL) to 0.1 µg/mL with **Dilution Buffer** to make **Streptavidin-HRP Working Solution.**

8.2. 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. Incubated 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 1M sulfuric acid, but the Signal-to-Background ratio may be reduced.

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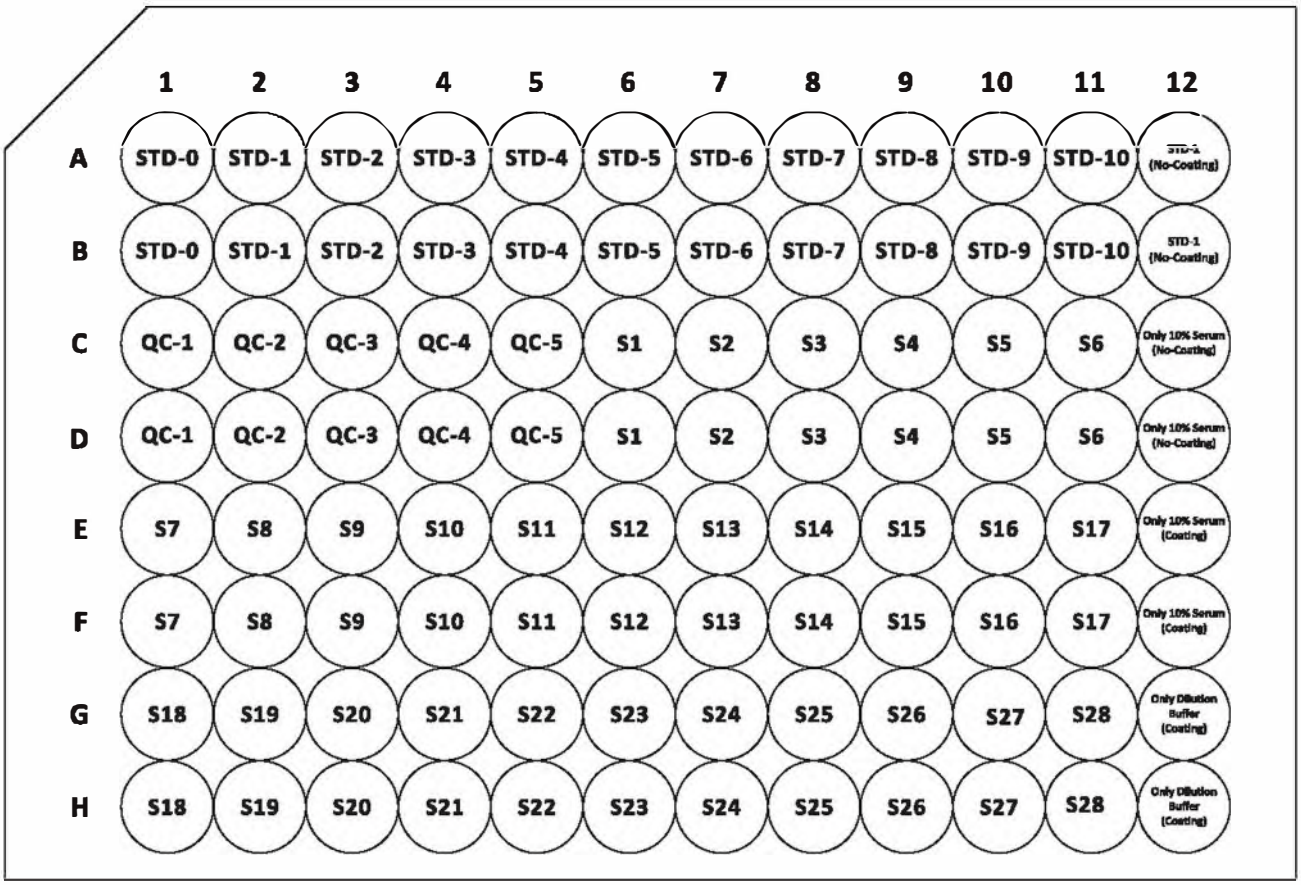
Fig.1 Plate Layout



Tab.3 Assay Protocol

Steps Code	Steps	Reagents & Instruments	Reaction Conditions	Samples/STDs /QCs	STD-1 (No-Coating)	Only 10% Serum (No-Coating)	Only 10% Serum (Coating)	Only Dilution Buffer (Coating)
1	Preparation	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2	Coating	Human CTLA-4 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	300 µL
4	Blocking	Blocking Buffer	37℃ for 1.5 hours	300 µL	300 µL	300 µL	300 µL	300 µL
5	Washing	Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL	300 µL

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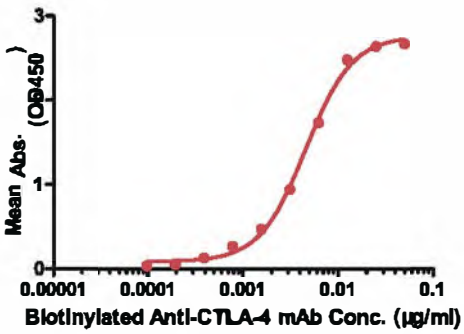
6	Add Samples	Mixed samples/ Mixed 1×STDs/ Mixed 1×QCs	-	100 µL	-	-	-	
		Mixed 1×STD-1	-	-	100 µL	-	-	-
		10% Serum	-	-	-	100 µL	100 µL	
		Dilution Buffer	-	-	-	-	-	100 µL
7	Washing	Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL	300 µL
8	Streptavidin-HRP Labeling	Streptavidin-HRP Working Solution	37℃ for 1 hour	100 µL	100 µL	100 µL	100 µL	100 µL
9	Washing	Wash Buffer	Wash for 3 times	300 µL	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	200 µL
11	Termination	Stop Solution	Mix by gentle tapping for 3 minutes	50 µL	50 µL	50 µL	50 µL	50 µL
12	Data Recording	UV/Vis spectrophotometer	Measure absorbance at 450 nm					

Notes: *It is recommended that all samples, controls and standards should be done in duplicates.*

Method Verification

- **CTLA-4: Biotinylated Anti-CTLA-4 Antibody Binding in the Absence of Competitors**
  Immobilized human **CTLA-4 Protein** at 1 µg/mL (100 µL/well) can bind the **Biotinylated Anti-CTLA-4 Antibody** with a linear range of 0.391-12.5 ng/mL when detected by **Streptavidin-HRP**. Background was subtracted from data points before curve fitting.



Sample Con. (µg/ml)	Mean Abs (OD450)
0.05	2.659
0.025	2.629
0.0125	2.471
0.00625	1.728
0.003125	0.939
0.001563	0.472
0.000781	0.268
0.000391	0.132
0.000195	0.052
9.77E-05	0.037
Blank	0.065

Fig.2 Binding of the biotinylated anti-CTLA-4 antibody to immobilized human CTLA-4 in a functional ELISA assay.

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- **Inhibition of CTLA-4: Biotinylated Anti-CTLA-4 Antibody Binding by an Anti-CTLA-4 Antibody**
  Serial dilutions of an **Anti-CTLA-4 Antibody** (Catalog # PK904-213) (1:2 serial dilutions, from 20 μg/mL to 0.039 μg/mL) was added into CTLA-4: **Biotinylated Anti-CTLA-4 Antibody** 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.

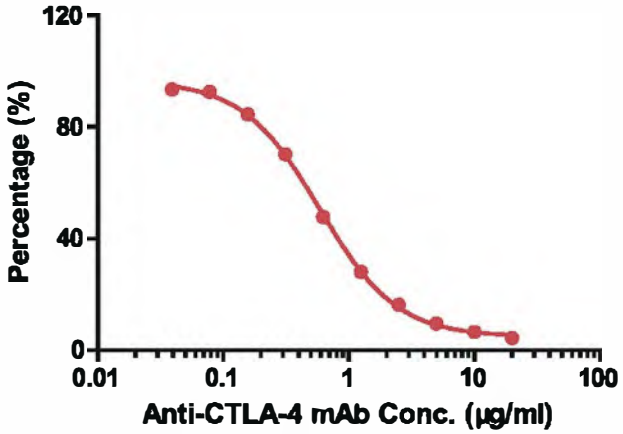

Fig.3 Inhibition of CTLA-4 : biotinylated anti-CTLA-4 antibody binding by an anti-CTLA-4 antibody.

Sensitivity: The lowest detectable level that can be distinguished from the zero standard is 0.15625 μg/mL.
Detection Range: 0.15625-5 μg/ml.
Precision:
Intra-assay CV: <20% for both calibration and QC points range 5- 0.15625 μg/mL for test run. Relax to CV < 25% and RE < 25% at the LLOQ (lower limit of quantification) and ULOQ (upper limit of quantification); for QC samples at high/low end use the same < 25% rule.
Inter-assay CV: < 20% for both calibration and QC points range 5- 0.15625 μg/mL for test run. Relax to CV < 25% and RE < 25% at the LLOQ (lower limit of quantification) and ULOQ (upper limit of quantification); for QC samples at high/low end use the same < 25% rule.
Recovery: Recovery rate was found to be between 80-120% with normal monkey serum samples with known concentrations.
Note: All final data should be within the detection range as described above.

Troubleshooting Guide
In case of a failed experiment, please check the expiration dates and status of the individual reagents, and make sure that all reagents have been reconstituted and stored as recommended. In addition, please make sure that all equipments are functioning properly.
Below is a list of common problems, and some tips that may help solve the problems and improve your results.
If you have any questions, please contact our technical support team at: ***PK@acrobiosystems.com***

Tab. 4 Troubleshooting Guide

Problem	Possible Cause	Solutions
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

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		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 low	▪ The concentration of the samples should be adjusted to achieve optimal r eading. ▪ Increase colorimetric HRP substrate incubation time
Signal of positive control is weak or abnormal	Human CTLA-4, human anti-CTLA-4 antibody,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 incorre ct; Incubation time is not sufficient	▪ Make sure the Substrate Stock Solution is working. ▪ Use proper incubation time and temperature.
	Insufficient mix	▪ Make sure the sample mixed sufficient before add to the plate
	Pipetting errors	▪ Make sure that the pipette is calibrated and working properly.
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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