RES59-EN.01

resDetect™ VH3 ELISA Kit (Residue Testing)

Catalog Number：RES-A059

Pack Size: 96 tests

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

For Research Use Only. Not For Use in Diagnostic or Therapeutic Procedure

INTENDED USE

The resDetect™ VH3 ELISA Kit (Residue Testing) is a complete kit for the quantitative determination of recombinant VH3 binding protein A. It is compatible to almost all VH3 resinsin the market. The resDetect™ VH3 ELISA Kit (Residue Testing) provides an effective sample treatment method to dissociate VH3 from IgG samples without boiling and centrifugation step. These solve the previous problem of poor recovery in some sample detection. At the same time, the procedure has been simplified with over 1 hour eliminated from sample treatment time.

BACKGROUND

Traditional protein A have affinity for both the Fc region and the Fab VH3 region of human antibodies. But VH3 interacts only with the variable heavy chain of the VH3 sequence family of the human antibody， it can be widely used in the purification of related antibody drugs.. However, during the purification , VH3 may leach from the purification column and resulte in contamination of the antibody drugs prepared. Therefore, the detection of residual VH3 in antibody drugs purified from VH3 purification column is a key quality control step in the production process of antibody drug preparations.

PRINCIPLE OF THE ASSAY

The resDetect™ VH3 ELISA Kit (Residue Testing) is used to measure the levels of recombinant VH3 by employing a standard sandwich-ELISA format. The micro-plate in the kit has been pre-coated with anti- VH3 polyclonal antibody. Firstly, the standard samples provided in kit and your samples are treated with Denaturation Buffer to dissociation of VH3 and antibody, stand a few minute. Before adding standards and samples, add the Biotin-Anti- VH3 Antibody to the plate to ensure that the standard samples are neutralized by the Biotin-Anti- VH3 Antibody buffer solution and protect the pre-coated antibody on the plate. Then, add the standard samples and your samples to the plate and form Antibody-antigen (VH3) - biotinylated antibody complex, incubate and wash the wells. Next add Horseradish peroxidase conjugated streptavidin (Streptavidin-HRP) to the plate, incubate and wash the wells to remove any unbound reactants. At last, load the tetramethylbenzidine (TMB) substrate into the

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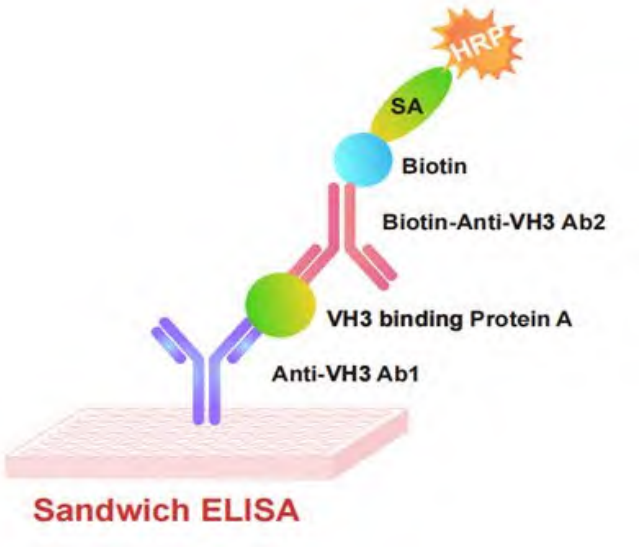
wells and monitor a blue color. The reaction is stopped by the addition of a stop solution and the color turns yellow. The intensity of the absorbance can be measured at 450nm and 630nm on a microtiter plate reader. The OD Value reflects the amount of VH3.



PRECAUTIONS

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.
2. Wear appropriate personal protective apparel, please be careful and avoid to contact the reagent with your skin, eyes and clothing. In case of accidental skin exposure, flush with water immediately. Consult a physician if required.
3. Do not use the kit and the all reagents past their expiration date.
4. Do not mix or substitute reagents with those from other kits or other lot number kits.
5. Activity of the conjugate is affected by nucleophiles such as azide, cyanide, and hydroxylamine.
6. If samples generate values higher than the highest standard, dilute the samples with the Dilution Buffer provided in kit and repeat the assay.
7. Differences in test results can be caused by a variety of factors, including laboratory operator, pipette usage, plate washing technique, reaction time or temperature, and kit storage.
8. This kit is designed to remove or reduce some endogenous interference factors in biological samples, and not all possible influencing factors have been removed.

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MATERIALS PROVIDED

Table1. Materials provided

Catalog	Components	Size (96 tests)	Format	Storage	
				Unopened	Opened
RES059-C01	Pre-Coated Anti- VH3 Antibody Microplate	1 plate	Solid	2-8℃	2-8℃
RES059-C02	Recombinant VH3 Standard (1μg/mL)	100 μL	Liquid	2-8℃	2-8℃
RES059-C03	Biotin-Anti- VH3 Antibody	1.5 mL	Liquid	2-8℃	2-8℃
RES059-C04	Streptavidin-HRP	10 μg	Powder	2-8℃, avoid light	-70℃, avoid light
RES059-C05	10×Sample Dilution Buffer	15 mL	Liquid	2-8℃	2-8℃
RES059-C06	Denaturation Buffer	15 mL	Liquid	2-8℃	2-8℃
RES059-C07	20×Washing Buffer	30 mL	Liquid	2-8℃	2-8℃
RES059-C08	Antibody Dilution Buffer	15 mL	Liquid	2-8℃	2-8℃
RES059-C9	Streptavidin-HRP Dilution Buffer	15 mL	Liquid	2-8℃	2-8℃
RES059-C10	Substrate Solution	12 mL	Liquid	2-8℃, avoid light	2-8℃, avoid light
RES059-C11	Stop Solution	8 mL	Liquid	2-8℃	2-8℃

Note: *It is recommended that Streptavidin-HRP be centrifuged briefly before use to deposit liquid from the tube wall or cap to the bottom of the tube.*

STORAGE

1. Unopened kit should be stored at 2℃-8℃ upon receiving.
2. Find the expiration date on the outside packaging and do not use reagents past their expiration date.
3. The opened kit should be stored per components table. The shelf life is 30 days from the date of

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opening.

REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED

1. Single or multi-channel micropipettes and pipette tips: need to meet 10 µL, 300 µL, 1000 µL injection requirements;
2. Orbital microtiter plate shaker：For shaking the plate in immunological steps.
3. Single or dual wavelength microplate reader with 450 nm and 630 nm filter;
4. Tubes: 1.5 mL,10 mL;
5. Timer;
6. Reagent bottle;
7. Deionized or distilled water.

REAGENT PREPARATION

1. Take out the kit, equilibrate all reagents and samples to room temperature (20℃-25℃) before use, check that each buffer and standard solution are clear and transparent, make sure these solution are evenly mixed.

Note: *RES059-C06 component does not clarify and RES059-C05 component is easy to crystallize at lower temperatures (2-8℃), so be sure to balance the two components at room temperature until the liquid is clarified.*

2. Reconstitute the provided lyophilized materials to stock solutions with sterile deionized water as recommended in the following table, Solubilize for 15 to 30 minutes at room temperature with occasional gentle mixing. Avoid vigorous shaking or vortex. The reconstituted stock solutions should be stored at -70℃. Avoid freeze-thaw cycles. It is recommended that the number of freezing and thawing should not exceed 1 time, the size of the aliquot should not be less than 5 µg.

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

Table 2. Preparation method

ID	Components	Size (96 T)	Storage solution concentration.	Reconstituted water Vol.
RES059-C04	Streptavidin-HRP	10 µg	100 µg/mL	100 µL water

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RECOMMENDED SAMPLE PREPARATION

1. Working Solution Preparation

1.1 Preparation of 1×Washing Buffer:

Dilute 25 mL 10×Washing Buffer with ultrapure water/deionized water to 500 mL. Please prepare it for one-time use only.

1.2 Preparation of 1×Sampie Dilution Buffer:

Dilute 10 mL 1×Sampie Dilution Buffer with ultrapure water/deionized water to 100 mL. Please prepare it for one-time use only.

1.3 Preparation of Biotin-Anti-VH3 Antibody working fluid:

Biotin-Anti-VH3 Antibody is diluted to a factor of 10 with Antibody Dilution Buffer (RES059-C08) according to the experimental dosage (100 µL/well). This working solution should be used immediately. Refer to Table 3 for configuration methods:

Table 3. Preparation method

Tests	Working solution	Biotin-Anti-VH3 Antibody	Antibody Dilution Buffer
96Tests	11000 µL	1100 µL	9900 µL

1.4 Preparation of Streptavidin-HRP working fluid:

The reconstructed Streptavidin-HRP storage solution was diluted to 0.15µg/mL by Streptavidin-HRP Dilution Buffer (RES059-C09) according to the experimental dosage (100µL/well). The prepared working fluid should avoid light.Please prepare it for one-time use only. Refer to Table 4 for configuration methods:

Table 4. Preparation method

Tests	Working solution	Streptavidin-HRP	Streptavidin-HRP Dilution Buffer
96Tests	11 mL	0.0165 mL	10.9835 mL

2. Preparation of Standard Curve

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This kit can be used for the quantitative detection of recombinant VH3 in neutral solution. The kit contains the standard of recombinant VH3 for establishing the standard curve.Each well requires 50 μL of standard according to the method.

Note: *Diluted standards should be used within 30 minutes of preparation.*

In order to counteract any standard sticking, we recommend changing tips between each dilution.

The recommended VH3 standard dilution procedure is listed and illustrated below:

2.1 Bring the VH3 standard stock solution to room temperature, the original concentration is 1 μg/mL.

2.2 Dilute the 1 μg/mL of standard stock solution -123fold with 1×Sample Dilution Buffer to 8.1 ng/mL (Std 6: 8.1ng/mL).

2.3 The standard curve is prepared by 3 times gradient dilution at the highest concentration point of the standard curve (Std 6:8.1 ng/mL), as shown below (taking the dilution volume of each concentration point of the standard product as 600μL). After each step of dilution, the remaining volume of the standard product should not be less than 0.1 ml;

- Add 400 μL of 1×Sample Dilution Buffer to each Std 5 to Std 2 tube;
- Add 200 μL of 1×Sample Dilution Buffer to tube of Std 1
- Add 200 μL Std 6 to 400μL 1×Sample Dilution Buffer, mix gently and repeat the serial dilution to make 5 VH3 standard solutions: Std 5, Std 4, Std 3, Std 2, Add 200 μL Std 2 to 400μL 1×Sample Dilution Buffer, mix gently and repeat the serial dilution to make standard solutions: Std 1
- Std 0 (Blank) is 1×Sample Dilution Buffer alone.

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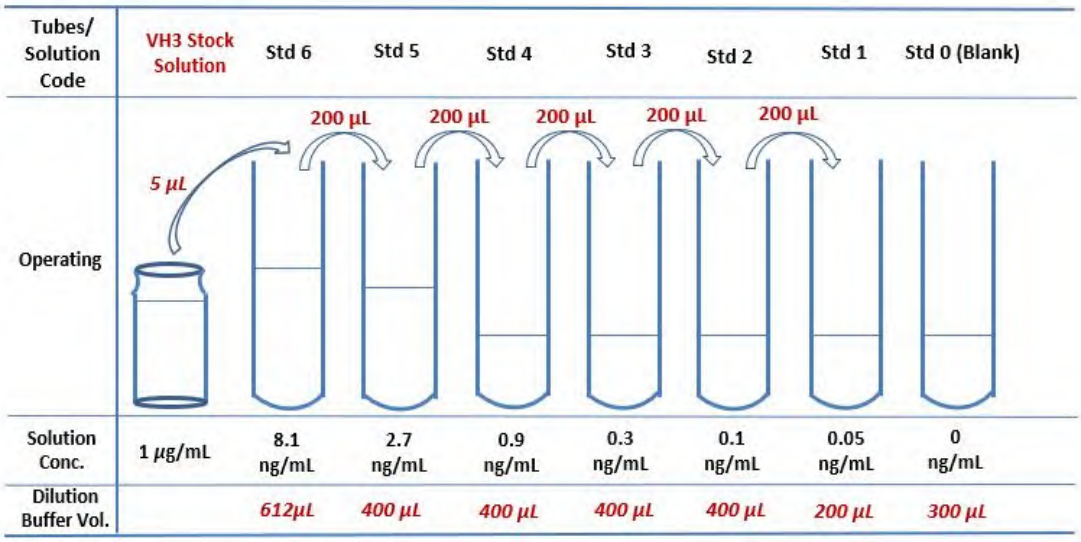

3. Prepare the Samples

Aliquot a minimum of 100 µL of each sample and standard into a microcentrifuge tube. Add 50 µL of Denaturation Buffer (RES059-C06) to each tube. Mix by pipetting up and down ~15 times or mix gently on a vortex mixer. Use fresh tips for each addition. Stand for 5-10 min.

Note: *The recovery rate of each testing sample shall be determined:*

1) All samples with a concentration of VH3 above the highest standard (Std 6) must be diluted, when the total amount of added VH3 and endogenous VH3 from the sample itself above the highest standard (Std 6), the samples also need to be diluted to a reasonable concentration, or your sample contains interfering ingredients, it also needs to be diluted to reduce interference.

2) When samples need to be diluted, dilute the samples with the 1×Sample Dilution Buffer to yield acceptable background and not impurities with VH3, sample dilution should be performed prior to the sample denaturation step for best results.

3) The diluted samples should also give acceptable recovery when spiked with known quantities of VH3, when the recovery rate is in the range of 80% to 120%, it indicates that the detection value of the diluted sample is reliable.This experiment can be performed by add a certain concentration of VH3 beyond the linear range to the samples, then dilute the sample to a reasonable range, this experiment

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also can be performed by spiking a standard provided with this kit with concentration in the linear range into the testing samples, for example, adding 1 part of the 0.8 ng/mL, 0.4 ng/mL or 0.2 ng/mL standard to 1 part of a 2mg/mL of test sample. This yields an added spike of 0.4 ng/mL, 0.2 ng/mL and 0.1 ng/mL, any endogenous VH3 from the sample itself determined prior to spiking and corrected for by the 50% dilution of that sample should be subtracted from the value determined for the spiked sample, then calculated the concentration of VH3 to give the recovery rate. If the VH3 content of the sample itself exceeds the highest standard (Std 6), dilute the sample to a linear concentration and then add standards for recovery: Refer to Table 5 for recovery test methods.

Table 5. Preparation method

Sample Recovery ID	Diluent Ratio	Sample and Standard Volume	Final Concentration of Sample	Final Concentration of VH3
Sample 1-R1	2	150 µL Standard 5 + 150 µL test sample	1 mg/mL	0.4 ng/mL
Sample 1-R2	2	150 µL Standard 4 + 150 µL test sample	1 mg/mL	0.2 ng/mL
Sample 1-R3	2	150 µL Standard 3 + 150 µL test sample	1 mg/mL	0.1 ng/mL
Sample 2-R1	4	150 µL Standard 5 + 150 µL Sample 1	0.5 mg/mL	0.4 ng/mL
Sample 2-R2	4	150 µL Standard 4 + 150 µL Sample 1	0.5 mg/mL	0.2 ng/mL
Sample 2-R3	4	150 µL Standard 3 + 150 µL Sample 1	0.5 mg/mL	0.1 ng/mL

4. Add Biotin-Anti- VH3 Antibody Working Solution and Samples

Add 100µL Biotin-Anti- VH3 Antibody working solution to each well, then add 50µL samples or standards to each well.

***Note**: All standards and samples to be tested should be on the same board and treated in the same way.*

5. Incubation

Seal the plate with microplate sealing film and incubate at room temperature (20℃-25℃) for 1 hour on orbital shaker at 400-600 rpm.

6. Washing

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Thorough washing is essential to proper performance of this assay. Automated plate washing systems or manual wash procedure be selected according to your own experimental conditions.

Remove the remaining solution of the wells, wash the wells by add 300 µL of 1×Washing Buffer to each well, gently tap the plate for 1 min, remove any remaining 1×Washing Buffer by aspirating or decanting, invert the plate and blot it against lint free paper towels to remove any remaining wash buffer. Please note that the complete removal of the washing buffer is essential.

Repeat the wash step above for 3 times.

7. Add Streptavidin-HRP Solution

Each well requires 100 µL of Streptavidin-HRP working solution.

8. Incubation

Seal the plate with microplate sealing film and incubate at room temperature (20℃-25℃) for 30min on orbital shaker at 400-600 rpm.

9. Washing

Repeat step 6.

10. Substrate Reaction

Add 100 µL Substrate Solution to each well. Seal the plate with microplate sealing film and incubate at room temperature (20℃-25℃) for 20 min, avoid light. Do not shake.

11. Termination

Add 50 µL Stop Solution to each well and tap the plate gently 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 and 630 nm using UV/Vis microplate spectrophotometer within 5 minutes.

Note*: To reduce the background noise, subtract the value read at* OD_{450nm} *with the value read at* $OD_{630\ nm}$.

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CALCULATION OF RESULTS

1. Calculate the mean absorbance for each standard, control and sample and subtract average zero standard optical density (OD).

2. The standard curve is plotted with the standard concentration as x-axis and the calibrated absorbance value as y-axis. Four parameters logistic are used to draw the standard curve and calculate the sample concentration. The concentration is calculated by multiplying it by the corresponding dilution.

3. Normal range of Standard curve: $R^2 \geq 0.9900$.

4. Detection range: 0.05 ng/mL-8.1 ng/mL. If the OD value of the sample to be tested is higher than the highest standard (8.1ng/mL), the sample shall be diluted with dilution buffer and assay repeated. If the OD value of the sample to be tested is lower than 0.05 ng/mL, the sample residual should be reported＜ 0.05 ng/mL.

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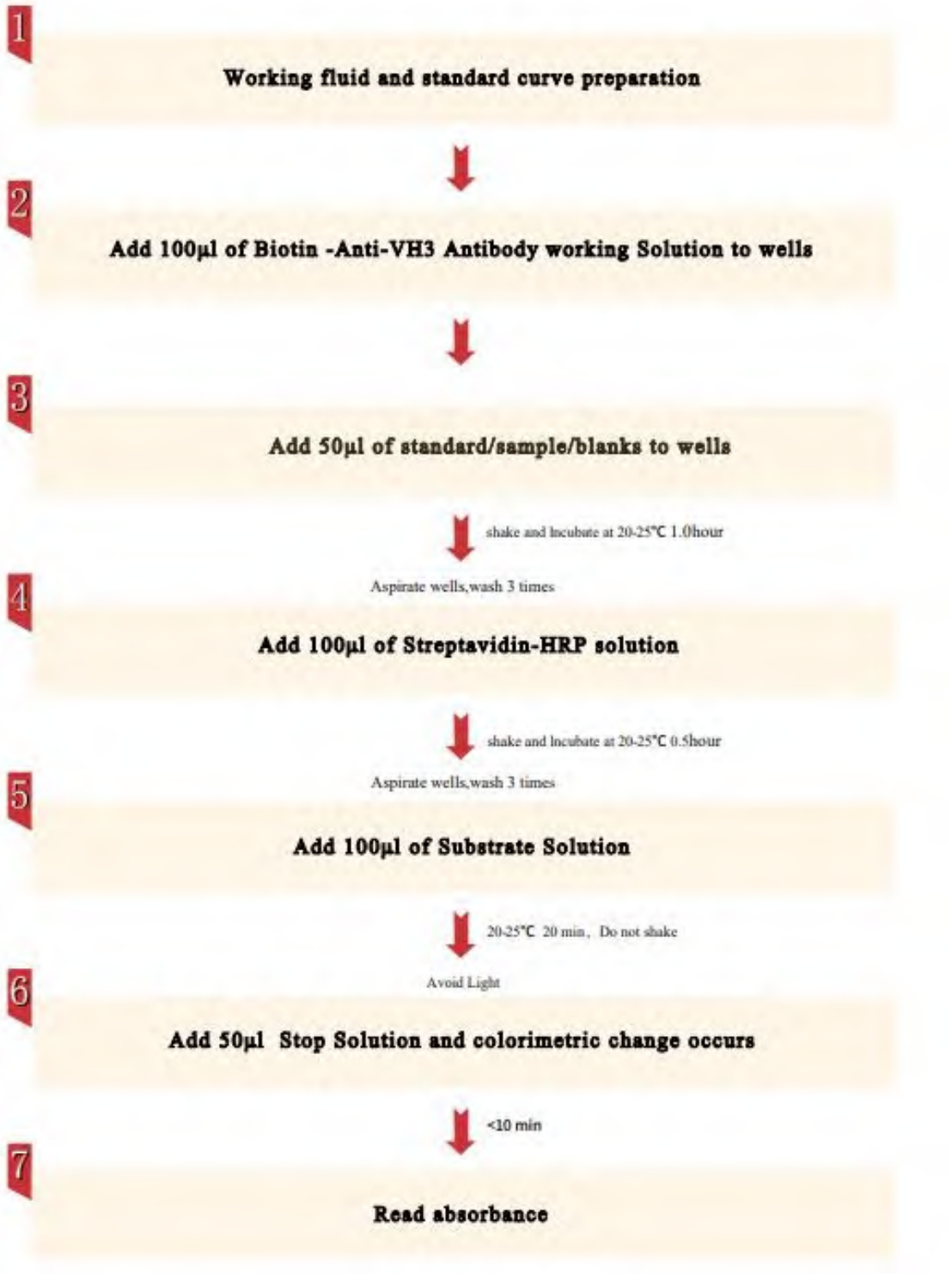
QUICK GUILD



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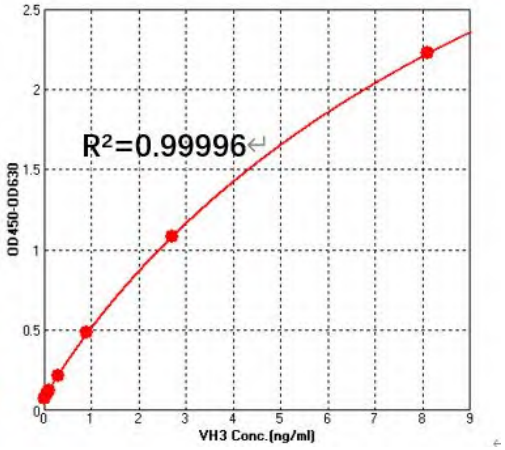
TYPICAL DATA

For each experiment, a standard curve needs to be set for each micro-plate, and the specific OD value may vary depending on different laboratories, testers, or equipments. The following example data is for reference only.

Standard curve of Recombinant VH3 binding Protein A:

Standard Num.	Concentration	$OD_{450nm-630nm}$
Standard 6	8.1 ng/mL	2.23
Standard 5	2.7 ng/mL	1.08
Standard 4	0.9 ng/mL	0.49
Standard 3	0.3 ng/mL	0.22
Standard 2	0.1 ng/mL	0.12
Standard 1	0.05 ng/mL	0.10
Standard 0	0 ng/mL	0.08



SENSITIVITY

The minimum detectable concentration of VH3 ELISA Kit is 0.04 ng/mL. Sensitivity (LOD) .

PRECISION

1. Intra-assay Precision

Three samples of known concentration were tested ten times on one plate to assess intra-assay precision.

2. Inter-assay Precision

Three samples of known concentration were tested in ten separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	10	10	10	10	10	10
Mean (ng/mL)	6.87	0.89	0.056	7.75	1.01	0.06

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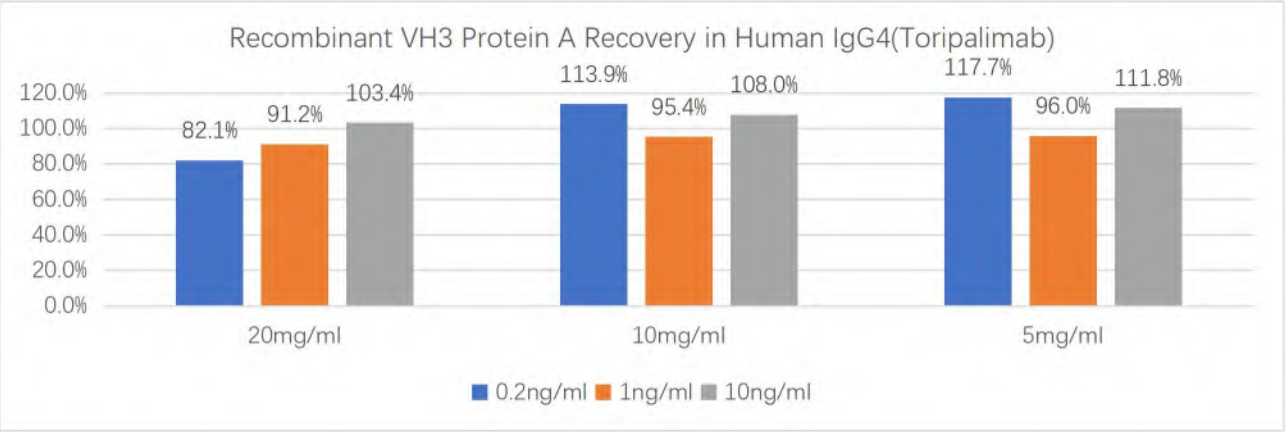
SD	0.084	0.028	0.004	0.104	0.023	0.004
CV (%)	3	4	3	4	4	4
Recovery（%）	85	99	114	96	112	120

***Note**: The example data is for reference only.*

RECOVERY

Add different concentrations of VH3 (0.2 ng/mL, 1 ng/mL, 10 ng/mL) to different concentrations of Human IgG4 (Toripalimab) (20 mg/mL, 10 mg/mL, 5 mg/mL), then dilute the antibodies to a reasonable range, then test and calculated the concentration of VH3 to give the recovery rate.

Add Recombinant VH3 to Human IgG4 (Toripalimab):



INTERFERING SUBSTANCES

We have conducted interference effect test about frequently-used buffers, they have excellent buffer compatibility. For specific buffers, it is recommended that you verify recovery to determine the minimum dilution ratio.

	Recombinant VH3	
Matrix	**Recovery（%）**	**Dilution Factor**
20mM L-histidine with 0.1% (w/v) PF68, pH6.0	100.9	1

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20mM L-histidine with 0.4% (w/v) Tween-80, pH6.0	114	1
1×PBS, pH7.3	86	1
1*PBS, pH7.3 with 11% Trehalose	91	2
20mM L-histidine, pH6.0	85	1
50mM Tris,100mM Glycine, pH7.5	81	1
100mM Tris,20mM Sodium citrate, pH7.5	85	1
20mM L-histidine 10% trehalose,pH6.0	99	2
50 mM Na Acetate, pH 3.5	98	1
25 mM Phosphate, pH 7.5	88	2
100 mM Glycine, pH 3.5	89	1
100 mM Triscitrate, 7.5	95	1
100 mM Trisacetate, 7.5	94	1

SPECIFICITY

Host cell protein (HCP 500 ng/mL) and host cell DNA (HCD 0.5 ng/mL) of HEK293, E.coli or CHO systems were added to human IgG1 (Bevacizumab, 1mg/mL) and human IgG4 (Toripalimab, 1mg/mL), respectively, which were higher than the usual quality standard limit. Then 2.7ng/mL、0.8ng/mL、0.05ng/mL of VH3 were added, respectively, and the ratio of Protein A recovery in the Protein A added samples without HCP and HCD was added as the specificity verification index. The calculation formula was as follows: (S3-S1) / (S2-S1) × 100%, the experimental design is as follows:

ID	Sample ID	Antibody Conc.(mg/mL)	VH3 (ng/ml)	HCP Conc.(ng/mL)	HCD Conc.(ng/mL)
S1	S1	1	0	0	0
S2	S2-1	1	3.2	0	0
	S2-2	1	0.3	0	0

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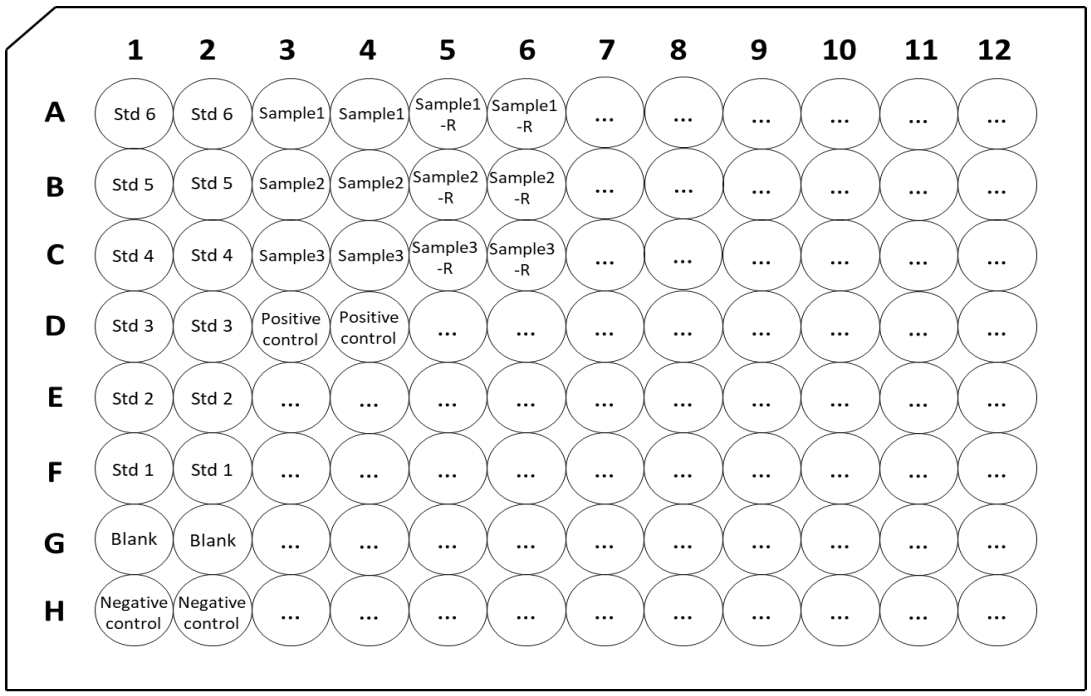
	S2-3	1	0.05	0	0
S3	S3-1	1	3.2	500	0.5
	S3-2	1	0.3	500	0.5
	S3-3	1	0.05	500	0.5

The results are as follows:

Sample	Antibody Conc. (mg/mL)	VH3 Conc. (ng/mL)	HCP Conc. (ng/mL)	HCD Conc. (ng/mL)	Bevacizumab			Toripalimab		
					HEK 293	E.coli	CHO	HEK 293	E.coli	CHO
Specificity of (VH3) Recovery	1	2.7	500	0.5	98%	97%	98%	95%	93%	110%
	1	0.8	500	0.5	84%	108%	87%	90%	94%	109%
	1	0.05	500	0.5	90%	103%	103%	83%	110%	104%

PLATE LAYOUT

It is recommended to use this flat layout to record standards and samples



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TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Poor standard curve	* Inaccurate pipetting	* Check pipettes
Large CV	* Inaccurate pipetting * Air bubbles in wells	* Check pipettes * Remove bubbles in wells
High background	* Plate is insufficiently washed * Contaminated wash buffer	* Review the manual for proper wash. * Make fresh wash buffer
Very low readings across the plate	* Incorrect wavelengths * Insufficient development time	* Check filters/reader * Increase development time
Samples are reading too high, but standard curve looks fine	* Samples contain cytokine levels above assay range	* Dilute samples and run again
Drift	* Interrupted assay set-up * Reagents not at room temperature	* Assay set-up should be continuous - have all standards and samples prepared appropriately before commencement of theassay * Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts

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