distributed in the US/Canada by:
EAGLE BIOSCIENCES, INC.
20A NW Blvd, Suite 112 Nashua, NH 03063
Phone: 617-419-2019 FAX: 617-419-1110
www.EagleBio.com info@eaglebio.com

KRIBIOLISA™ Vero HCP ELISA

REF : KBBP16

Ver 1.1

RUO

Enzyme Immunoassay for the Quantitative Determination of Vero Host Cell Proteins in cell culture supernatant and biological solutions

RUO	**For Research Use Only**	**REF**	**Catalog Number**
	Store At	**LOT**	**Batch Code**
	Manufactured By		**Biological Risk**
	Expiry Date		**Consult Operating Instructions**

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REF KBBP16 Σ 96 tests

KRISHGEN BioSystems For US/Europe Customers: toll free +1(888)-970-0827 | tel +1(562)-568-5005
For Asia/India Customers: +91(22)-49198700
Email: sales@krishgen.com | http://www.krishgen.com

Introduction:

A variety of proteins which are used as therapeutic agents in humans and animals are produced through recombinant expression in VERO cells. The manufacturing and purification process of these products tends to leave the potential for contamination by Host Cell Proteins (HCPs) from VERO cells, which may result in adverse toxic or immunological reactions that ultimately affect the efficacy of the therapeutic agent. The simple, objective and semi-quantitative ELISA is a highly sensitive method that aids in purification process development, process control, quality control and product release testing optimally.

Intended Use:

This generic kit is intended in determining the presence Vero Host Cell Proteins contamination in various products that are manufactured through recombinant expression in VERO cells. The kit has been validated successfully for testing of in process and final product HCPs in variety of products regardless of growth and purification process.

Principle:

The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and Vero HCP present in the sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin-HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Vero HCP in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Materials Provided:

1. Vero HCP Antibody Coated Microtiter Plate (8 x 12 wells) - 1 no
2. Vero HCP Standard (lyophilized, concentrated, 20 ng/ml) - 2 vials
3. Biotinylated Vero HCP Antibody (concentrated) - 120 ul
4. Streptavidin:HRP Conjugate (concentrated) - 120 ul
5. Sample Diluent 1 - 20 ml
6. Sample Diluent 2 - 20 ml
7. Biotin Antibody Dilution Buffer - 10 ml
8. HRP Conjugate Dilution Buffer - 10 ml
9. (20X) Wash Buffer - 25 ml
10. TMB Substrate - 12 ml
11. Stop Solution - 12 ml
12. Instruction Manual

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul
3. Deionized (DI) water
4. Wash bottle or automated microplate washer
5. Clean tubes and Eppendorf tubes
6. Precision single and multi-channel pipette and disposable tips.
7. 37°C incubator
8. Timer.

Handling/Storage:

1. All reagents should be stored as indicated on the component label.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.

3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

Sample Preparation and Storage:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

1. **Cell Culture Supernatant-** Centrifuge supernatant for 20 minutes at 1000×g at 2-8°C to remove insoluble impurity and cell debris. Collect the clear supernatant and carry out the assay immediately.
2. **Cell Culture Lysate**: Commercial RIPA kits are recommended to follow the instructions provided. Generally, 0.5 ml RIPA lysis buffer would be appropriate to $2x10^6$cells, DNA must to be removed. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3 mg.
3. **Other Biological Fluids**: Centrifuge samples for 20 minutes at 1000×g at 2-8°C. Collect supernatant and carry out the assay immediately.
   Note: Samples to be used within 5 days can be stored at 2-8°C, besides that, samples must be stored at -20°C (assay ≤ 1 month) or -80°C (assay ≤ 2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles. The hemolytic samples are not suitable for this assay.

Sample Dilution

Please refer to the following table of recommended dilution ratio for Vero samples for reference.

Dilution Fold	Sample	Sample Diluent 1	Sample Diluent 2	Total Diluted Sample Volume
1/2	60 ul	60 ul	---	120 ul
1/5	24 ul	96 ul	---	120 ul
1/10	12 ul	108 ul	---	120 ul
1/20	6 ul	114 ul	---	120 ul
1/50	3 ul	---	47 ul	50 ul + 100 ul Sample Diluent 1
1/100	3 ul	---	177 ul	180 ul + 120 ul Sample Diluent 1
1/1000	2 step dilution. Create a 50 fold dilution and then make a 20 fold dilution. Sample diluent 2 is used throughout the dilution.			
1/10000	2 step dilution. Create a 100 fold dilution and then make a 100 fold dilution using Sample diluent 2 is used throughout the dilution.			
1/100000	3 step dilution. Create a 50 fold dilution and then make a 20 fold dilution. Finally create a 100 fold dilution using Sample diluent 2 is used throughout the dilution.			

Note: The volume in each dilution is not less than 3 ul. Dilution factor should be within 100 fold. Mix well during dilution and avoid foaming.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make **Wash Buffer (1X);** dilute **25 ml of (20X) Wash Buffer in 475 ml of DI water**.
4. **Biotinylated Vero HCP Antibody Working Solution**: Prepare it within 1 hour before experiment. Calculate required total volume of the working solution: 0.1 ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the total volume. Dilute the Biotinylated Vero HCP Antibody (concentrated) with Biotin Antibody Dilution Buffer at 1:100 and mix them thoroughly (i.e. Add 1 ul Biotinylated Vero HCP Antibody into 99 ul Biotin Antibody Dilution Buffer).
5. **Streptavidin:HRP Conjugate Working Solution**: Prepare it within 30 minutes before experiment. Calculate required total volume of the working solution: 0.1ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the

total volume. Dilute the Streptavidin:HRP Conjugate with Streptavidin:HRP Conjugate Dilution Buffer at 1:100 and mix them thoroughly (i.e. Add 1 ul of Streptavidin:HRP Conjugate into 99 ul of Streptavidin:HRP Conjugate Dilution Buffer).

6. **Standards Preparation**: Reconstitute original Vero HCP Standard with 1 ml of Sample Diluent1. Keep the standard for 15 mins with gentle agitation before making further dilutions. Prepare the additional Standards by serially diluting the standard stock solution as per the below table.

Standard Concentration	Standard Vial	Dilution Particulars
20 ng/ml	Standard No.8	Reconstitute with 1 ml Sample Diluent1
10 ng/ml	Standard No.7	300 ul Standard No.8 + 300 ul Sample Diluent1
5 ng/ml	Standard No.6	300 ul Standard No.7 + 300 ul Sample Diluent1
2.5 ng/ml	Standard No.5	300 ul Standard No.6 + 300 ul Sample Diluent1
1.25 ng/ml	Standard No.4	300 ul Standard No.5 + 300 ul Sample Diluent1
0.625 ng/ml	Standard No.3	300 ul Standard No.4 + 300 ul Sample Diluent1
0.312 ng/ml	Standard No.2	300 ul Standard No.3 + 300 ul Sample Diluent1
0 ng/ml	Standard No.1	300 ul Sample Diluent1 only

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Vero HCP. High Dose Hook Effect is due to excess of antibody for very high concentrations of Vero HCP present in the sample.
3. Vero HCP concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
4. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Vero HCP.
5. It is recommended that all Standards and Samples be assayed in duplicates or triplicates.
6. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
7. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
8. The plates should be read within 30 minutes after adding the Stop Solution.
9. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.

2. Add **100 ul prepared Standards and diluted Samples** to respective wells.

3. Cover the plate with a sealer and incubate for 90 minutes at 37°C.

4. Aspirate and wash plate 2 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

5. Pipette **100 ul Biotinylated Vero HCP Antibody Working Solution** to all wells.

6. Cover the plate with a sealer and incubate for 60 minutes at 37°C.

7. Aspirate and wash plate 4 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step

8. Pipette **100 ul Streptavidin:HRP Conjugate Working Solution** to all wells. Mix well.

9. Cover the plate with a sealer and incubate for 30 minutes at 37°C.

10. Aspirate and wash plate 5 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step

11. Pipette **100 ul TMB Substrate** in all the wells.

12. Incubate the plate at **37°C** for **20 minutes**. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

13. Pipette **100 ul** of **Stop Solution** to all wells. The wells should turn from blue to yellow in color.

14. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples after subtracting the zero standard (blank) absorbance values. Using Graph Paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Vero HCP concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Vero HCP Concentration.

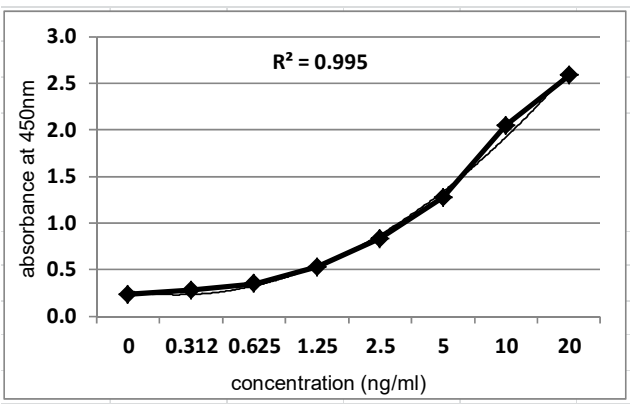
If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4-PL (2nd order) is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.

Typical Data

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration	% STD Deviation	CV	%CV	Net Signal Difference
0	0.254	0.217	0.236		--	2.6	0.1	11.0	0.000
0.312	0.275	0.288	0.282	0.3	87.7	0.9	0.0	3.3	0.046
0.625	0.347	0.351	0.349	0.6	94.6	0.3	0.0	0.9	0.067
1.25	0.536	0.524	0.530	1.3	107.6	0.9	0.0	1.6	0.181
2.5	0.820	0.852	0.836	2.6	104.1	2.3	0.0	2.7	0.306
5	1.301	1.243	1.272	4.7	93.4	4.1	0.0	3.2	0.436
10	2.138	1.952	2.045	10.5	104.9	13.1	0.1	6.4	0.773
20	2.608	2.559	2.583	19.6	98.0	3.5	0.0	1.3	0.538

Typical Graph



Abs = absorbance at 450nm

Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

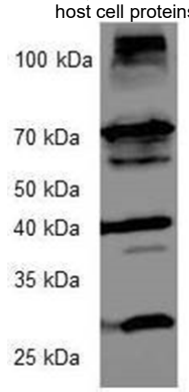
Performance Characteristics of the Kit:
This kit has been validated. Please view the details herein below.

Standard Calibration Range:
0.312 ng/ml - 20 ng/ml

Sensitivity:
Limit of Quantification: It is defined as the lowest detectable concentration that can be determined with an acceptable repeatability and the LOQ was found to be 0.20 ng/ml.

Specificity:
This assay has high sensitivity and excellent specificity for detection of VERO HCP. No significant cross-reactivity or interference between VERO HCP and analogues was observed. The antigen used was developed from mock fermented VERO media. The western blot was done to view the coverage of the HCP proteins. (picture below).

Vero cell lystates coverage of host cell proteins



The antibodies developed against the purified antigen are rabbit polyclonals affinity purified.

Recovery
Matrices listed below were spiked with certain level of Vero HCP and the recovery rates were calculated by comparing the measured value to the expected amount of Vero HCP in samples.

Sample Buffer Matrix	Pure Antigen Added (ng/ml)	Observed	Recovery (%)
Cell Culture Supernatant	0.625	0.56	90%
	5	5.75	115%
	20	22.0	110%
0.1M PBS Diluent	0.625	0.65	104%
	5	4.80	96%
	20	20.4	102%

Precision
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (0.312 ng/ml) and high (20 ng/ml) concentrations. While actual precision may vary from laboratory to and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results:

Pool	Intra Assay %CV	Inter Assay %CV
Low	~11.05%	~8.65%
High	~9.8%	~9.70%

Linearity

Dilute the sample with a certain amount of VERO HCPs at 1:2, 1:4 and 1:8 to get the recovery range.

Matrix	1:2	1:4	1:8
Cell Culture Supernatant (n=10)	96-106.5%	97-108%	87-114%

Safety Precautions:

- **This kit is For Research Use only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
- Do not smoke, eat or drink while handling kit material
- Always use protective gloves
- Never pipette material by mouth
- Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	Interpolated Concentration
1A 2A	Standard No.1 Standard No.1			
1B 2B	Standard No.2 Standard No.2			
1C 2C	Standard No.3 Standard No.3			
1D 2D	Standard No.4 Standard No.4			
1E 2E	Standard No.5 Standard No.5			
1F 2F	Standard No.6 Standard No.6			
1G 2G	Standard No.7 Standard No.7			
1H 2H	Standard No.8 Standard No.8			
3A 4A	Sample Sample			
3B 4B	Sample Sample			

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SYMBOLS KEY

MTP	Coated Microtiter Plate (8x12 wells)
STD	Standard
BIOTIN AB	Biotinylated Antibody
HRP CONJ	Conjugate Horseradish Peroxidase
BIOTIN DIL	Biotin Antibody Dilution Buffer
HRP DIL	HRP Conjugate Dilution Buffer
SAMP DIL 1	Sample Diluent 1
SAMP DIL 2	Sample Diluent 2
20X WASH BUF	(20X) Wash Buffer
SUB TMB	TMB Substrate
SOLN STOP	Stop Solution
	Consult Instructions for Use
REF	Catalog Number
	Expiration Date
	Storage Temperature