Version 26-12/24

User's Manual

S.cerevisiae HCP ELISA kit

REF **DEIABL494**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

This kit is intended for use in determining the presence of *Saccharomyces cerevisiae* protein contamination in products manufactured by recombinant expression in this yeast cell line. The kit is for Research and Manufacturing Use Only and is not intended for diagnostic use in humans or animals.

General Description

Recombinant expression of proteins by *S.cerevisiae* is an efficient method to obtain cost effective quantities of a desired protein. Many of these recombinantly produced proteins are intended for use as therapeutic agents in humans and animals and as such must be highly purified. The manufacturing and purification process of these products leaves the potential for contamination by host cell proteins from *S. cerevisiae*. Such impurities can result in adverse toxic or immunological reactions and thus it is desirable to reduce host cell impurity to the lowest levels practical.

Immunological methods using antibodies to HCPs such as Western Blot and ELISA are conventionally accepted. While Western blot is a useful method aiding in the identity of HCPs, it suffers from a number of limitations. Western blot is a complex and technique dependent procedure requiring a subjective interpretation of results. Furthermore, it is essentially a qualitative method and does not lend itself to obtaining quantitative answers. The sensitivity of Western blot is severely limited by the volume of sample that can be tested and by interference from the presence of high concentrations of the intended product. Western Blot may be able to detect HCPs in samples from upstream in the purification process but it often lacks adequate sensitivity and specificity to detect HCPs in purified downstream and final product. The microtiter plate immunoenzymetric assay (ELISA) method employed in this kit overcomes the limitations of Western blots providing on the order of 100 fold better sensitivity. This simple to use, highly sensitive, objective, and semiquantitative ELISA is a powerful method to aid in optimal purification process development, process control, routine quality control, and product release testing. This kit is "generic" in the sense that it is intended to react with essentially all of the HCPs that could contaminate the product independent of the purification process. The antibodies have been generated against and affinity purified using a mild lysate washed of *S. cerevisiae* cells to obtain HCPs typically encountered in your initial product recovery step. Western blot was used as a preliminary method and established that the antibodies reacted to the majority of HCP bands resolved by the PAGE separation. If you have need of a more sensitive and specific method to demonstrate reactivity to individual HCPs in your samples Creative Diagnostics recommends a method we find superior to 2D Western blot. We term this method 2D HPLC-ELISA. 2D HPLCELISA can yield much better sensitivity and specificity as compared to 2D Western blot. For more information on this 2D HPLC-ELISA analysis please contact our Technical Services department.

Special procedures were utilized in the generation of these antibodies to insure that low molecular weight and less immunogenic impurities as well as high molecular weight components would be represented. As such this kit can be used as a process development tool to monitor the optimal removal of host cell impurities as well as in routine final product release testing. Because of the high sensitivity and broad reactivity of the antibodies, this generic kit has been successfully qualified for testing of final product HCPs in many different products regardless of growth and purification process. When the kit can be satisfactorily qualified for your samples, the application of a more process specific assay is probably not necessary in that such an assay would only provide information redundant to this generic assay. However, if your qualification studies indicate the antibodies in this kit are not sufficiently reactive with your process specific HCPs it may be desirable to

also develop a more process specific ELISA. This later generation assay may require the use of a more specific and defined antisera. Alternatively, if the polyclonal antibody used in this kit provides sufficient sensitivity and broad antigen reactivity, it may be possible to substitute the standards used in this kit for ones made from the impurities that typically co-purify through your purification process and thus achieve better accuracy for process specific HCPs. The suitability of this kit for a given sample type and product must be determined and qualified experimentally by each laboratory. The use of a process specific assay with more defined antigens and antibodies in theory may yield better sensitivity however Saccharomyces cerevisiae HCP ELISA Product Insert 2 such an assay runs the risk of being too specific in that it may fail to detect new or atypical impurities that might result from some process irregularity or change. For this reason it is recommended that a broadly reactive "generic" host cell protein assay be used as part of the final product purity analysis even when a process specific assay is available. If you deem a more process specific assay is necessary, Creative Diagnostics is available to apply its proven technologies to develop such antibodies and assays on custom basis.

Principles of Testing

The *Saccharomyces cerevisiae* Host Cell Protein assay is a two-site immunoenzymetric assay. Samples containing *S. cerevisiae* proteins are reacted in microtiter strips coated with an affinity purified anti-*S. cerevisiae* capture antibody. A second biotinylated anti-*S. cerevisiae* antibody is reacted simultaneously forming a sandwich complex of solid phase antibody-*S. cerevisiae* protein-biotin labeled antibody. The microtiter strips are then washed to remove any unbound reactants. Streptavidin: Alkaline Phosphatase is added to bind to any biotinylated antibody bound to the well. The substrate p-nitrophenyl phosphate (PNPP) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and will be directly proportional to the concentration of *S. cerevisiae* proteins present.

Reagents And Materials Provided

Anti-*S. cerevisiae*, biotinylated

Affinity purified goat antibody conjugated to biotin in a protein matrix with preservative. 1x12mL

Anti-*S. cerevisiae* coated microtiter strips, 12x8 well strips in a bag with desiccant

***S. cerevisiae* Standards**

Solubilized *S. cerevisiae* HCPs in bovine albumin with preservative. Standards at 0, 2, 8, 25, 75, and 200ng/mL. 1 mL/vial

Streptavidin: Alkaline Phosphatase

In a protein matrix with preservative. 1x12mL.

PNPP Substrate

p-nitrophenyl phosphate in a Diethanolamine buffer with preservative. 1x12mL

Wash Concentrate (20X)

Tris buffered saline with preservative. 1x50mL

Materials Required But Not Supplied

1. Microtiter plate reader spectrophotometer with dual wavelength capability at 405 & 492nm. (If your plate

reader does not provide dual wavelength analysis you may read at just the 405nm wavelength.)

2. Pipettors - 50µL and 100µL
3. Repeating or multichannel pipettor - 100µL
4. Microtiter plate rotator (400 - 600 rpm)
5. Sample Diluent
6. Distilled water
7. 1 liter wash bottle for diluted wash solution

Storage

All reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.

The substrate reagent should not be used if its absorbance at 405nm is greater than 0.4.

Reconstituted wash solution is stable until the expiration date of the kit.

After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the "Reagent Preparation" section.

Reagent Preparation

Bring all reagents to room temperature.

Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Assay Procedure

Notes

1. Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. We advise against the use of automated or other manual operated vacuum aspiration devices for washing plates as these may result in lower specific absorbances, higher non-specific absorbance, and more variable precision. The manual wash procedure described below generally provides lower backgrounds, higher specific absorbance, and better precision. If duplicate CVs are poor or if the absorbance of the 0 standard is greater than 0.300, evaluate plate washing procedure for proper performance.
2. High Dose Hook Effect or poor dilutional linearity may be observed in samples with very high concentrations of HCP. High Dose Hook Effect is due to insufficient excess of antibody for very high concentrations of HCPs present in the samples upstream in the purification process. Samples greater than 50 µg/mL may give absorbances less than the 200 ng/mL standard. It is also possible for samples to have certain HCPs in concentrations exceeding the amount of antibody for that particular HCP. In such cases the absorbance of the undiluted sample may be lower than the highest standard in the kit however these samples will fail to show acceptable dilutional linearity/parallelism as evidenced by an apparent increase in dilution corrected HCP concentration with increasing dilution. High Dose Hook and poor dilutional linearity are most likely to be encountered from samples early in the purification process. If a hook effect is possible, samples should also be assayed diluted. If the HCP concentration of the undiluted sample is less than the diluted sample this may be indicative of the hook effect. Such samples should be diluted at least to the minimum required

dilutions (MRDs) as established by your qualification studies using your actual final and in-process drug samples. The MRD is the first dilution at which all subsequent dilutions yield the same HCP value within the statistical limits of assay precision. The HCP value to be reported for such samples is the dilution corrected value at or greater than the established MRD. The diluent used should be compatible with accurate recovery. The preferred diluent is available in 100mL, 500mL, or 1 liter bottles. This is the same material used to prepare the kit standards. As the sample is diluted in the diluent we recommend, its matrix begins to approach that of the standards thus reducing any inaccuracies caused by dilutional artifacts. Other prospective diluents must be tested for recovery by using them to dilute the 200ng/mL standard, as described in the "Limitations" section below.

3. If the substrate has a distinct yellow color prior to performing the assay it may have been contaminated. If this appears to be the case read 200µL of substrate against a water blank. If the absorbance is greater than 0.4 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised. The PNPP substrate is very sensitive to environmental impurities. Do not leave bottle open or at room temperature for longer than is needed. Only remove as much reagent as is needed for your assay run and do not return any unused substrate back into the substrate bottle.

Assay Protocol

1. The assay protocol is a simultaneous incubation of sample with biotinylated antibody. This yields a sensitivity of 0.5ng/mL and requires 4 hours to complete.
2. The assay is very robust such that assay variables like incubation times, sample size, and other sequential incubation schemes can be altered to manipulate assay performance for more sensitivity, increased upper analytical range, or reduced sample matrix interference. Before modifying the protocol from what is recommended, users are advised to contact our technical services for input on the best way to achieve your desired goals.
3. The protocol specifies the use of an approved orbital microtiter plate shaker for the immunological step. These can be purchased from most laboratory supply companies. If you do not have such a device it is possible to incubate the plate without shaking, however it will be necessary to extend the first immunological incubation step by one hour in order to achieve comparable results to the 2-hour shaking protocol. **Do not shake during the 1-hour substrate incubation step as this may result in higher backgrounds and worse precision.**
4. Bring all reagents to room temperature.
5. Set-up plate spectrophotometer to read dual wavelength at 405nm for the test wavelength and 492nm for the reference wavelength.
6. All standards, controls and test samples should be assayed at least in duplicate.
7. Maintain a repetitive timing sequence from well to well for all assay steps to insure that all incubation times are the same for each well.
8. Make a work list for each assay to identify the location of each standard control and patient.
9. Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy.

Operate as follows

1. Pipette 50µL of standards, controls and samples into wells indicated on work list.
2. Pipette 100µL of biotinylated anti-S. cerevisiae into each well.
3. Cover & incubate on orbital shaker at 400 – 600 rpm for 2 hours at room temperature, 24°C + 4°C.

4. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding substrate.
5. Pipette 100µL of Streptavidin: Alkaline Phosphatase into each well.
6. Cover & incubate on orbital shaker at 400 – 600 rpm for 1 hour at room temperature, 24°C + 4°C.
7. Repeat Step 4 as shown above.
8. Pipette 100µL of PNPP substrate.
9. Incubate for 1 hour at room temperature.
10. Read absorbance at 405/492nm.

Quality Control

1. Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 2 ng/mL and < 200 ng/mL. CVs for samples < 2 ng/mL may be greater than 10%.
2. For optimal performance the absorbance of the substrate when blanked against water should be < 0.4.
3. It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct. You are strongly urged to make controls in your typical sample matrix using HCPs derived from your cell line. These controls can be aliquoted into single use vials and stored frozen for longterm stability.

Calculation

The standards may be used to construct a standard curve with values reported in ng/mL "total immuno reactive HCP equivalents" (See Limitations section). This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, cubic spline, or 4 parameter logistic fit. Do not use linear regression analysis to interpolate values for samples as this may lead to significant inaccuracies! Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Typical Standard Curve

Well #	Contents	Abs. at 405-490nm	Mean Abs.
A1	Zero Std	0.000	0.002
B1	Zero Std	0.004	
C1	2ng/mL	0.025	0.024
D1	2ng/mL	0.023	
E1	8ng/mL	0.101	0.098
F1	8ng/mL	0.095	
G1	25ng/mL	0.306	0.300
H1	25ng/mL	0.295	
A2	75ng/mL	0.879	0.872
B2	75ng/mL	0.865	
C2	200ng/mL	1.702	1.712
D2	200ng/mL	1.722	

Performance Characteristics

Hook Capacity

Increasing concentrations of HCPs> 200 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 200 ng/mL standard was>50 µg/mL.

Precision

Both intra (n=20 replicates) and inter-assay (n=5 assays) precision were determined on 3 pools with low (2ng/mL), medium (75ng/mL), and high concentrations (200ng/mL). The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra assay CV	Inter assay CV
Low	15.4%	16.1%
Medium	4.2%	5.6%
High	2.1%	3.4%

Detection Limit

The lower limit of detection (LOD) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD is 0.5ng/mL.

The lower limit of quantitation (LOQ) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is 0.9ng/mL.

Specificity

1D Western blot analysis against other strains of *S. cerevisiae* indicates that most of the proteins are conserved among all strains. Thus, this assay should be useful for detecting HCP's from other Saccharomyces cerevisiae strains. Each end user must qualify that this kit is adequately reactive and specific for their samples. 1D Western blot is highly orthogonal to ELISA and to non-specific protein staining methods such as silver stain or colloidal gold. As such, the lack of identity between silver stain and western blot does not necessarily mean there is no antibody to that protein or that the ELISA will not detect that protein. If you desire a much more sensitive and specific method than Western blot to detect the reactivity of the antibodies in this kit to your individual HCPs CD is pleased to offer a service and/or consultation on fractionation of HCPs using 2 Dimensional HPLC methods followed by detection in the ELISA. This method has been shown to be much at least 100 fold more sensitive than Western blots in detecting antibody reactivity to individual HCPs. The same antibody as is used for both capture and HRP label can be purchased separately. Cell proteins from the yeast Pichia pastoris were tested and found to be non-reactive.

Recovery

Various buffer matrices were evaluated by adding known amounts of *S. cerevisiae* HCP preparation used to make the standards in this kit. Because this assay is designed to minimize matrix interference most of these buffers yielded acceptable recovery defined as between 80-120%. In general extremes in pH (8.5) as well as certain detergents can cause under-recovery. High concentrations of the recombinant product protein may also cause positive or negative interference in this assay. Each user should qualify that their sample matrices yield accurate recovery. Such an experiment can be performed, by diluting the 200ng/mL standard provided with this kit, into the sample matrix in question and assaying for recovery.

Precautions

1. For Research or Manufacturing use only.
2. At the concentrations used in this kit, none of the reagents are believed to be harmful.
3. This kit should only be used by qualified technicians

Limitations

1. Before relying exclusively on this assay to detect host cell proteins, each laboratory should qualify that the kit antibodies and assay procedure yield acceptable specificity, accuracy, and precision. A suggested protocol for this qualification can be obtained by contacting our Technical Services Department or at our web site.
2. The standards used in this assay are comprised of S. cerevisiae HCPs solubilized by mechanical disruption and detergent. 1D Western blot analysis of the antibodies used in this kit demonstrates that they recognize the majority of distinct PAGE separated bands seen using a sensitive protein staining method like silver

stain or colloidal gold. Because the vast majority of HCPs will be conserved among all strains of S. cerevisiae, this kit should be adequately reactive to HCPs from your strain. Other clients have successfully qualified this kit for their individual S. cerevisiae strains demonstrating acceptable specificity, accuracy, and sensitivity for process intermediate samples as well as final product. However, there can be no guarantee that this assay will detect all proteins or protein fragments from your process. If you desire a much more sensitive and specific method than western blot to detect the reactivity of the antibodies in this kit to your individual HCPs, Creative Diagnostics is pleased to offer a service for fractionation of HCPs using 2-D HPLC methods followed by detection in ELISA.

3. Certain sample matrices may interfere in this assay. However, the potential exists that the product protein itself, high or low pH, high salt, detergents, or other components in the sample matrix may result in either positive or negative interference in this assay. It is advised to test all sample matrices for interference by diluting the 200ng/mL standard, 1 part to 4 parts of the matrix containing no or very low HCP impurities. This diluted standard when assayed as an unknown should give a value of 30 to 50 ng/mL. Consult Creative Diagnostics Technical Service Department for advice on how to quantitate the assay in problematic matrices.

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