SARTORIUS

Host Cell Protein Detection

Technical Note

Scope
This technical note discusses how to customize a residual host cell protein (HCP) contaminant assay on Octet® R8, RH16 and RH96 BLI systems. It walks the reader through the critical steps of biotinylation of anti-HCP antibodies to be used with Streptavidin-based biosensors and the requisite reagents for the detection of HCPs through an amplified signal response assay. The approach can be applied to any cell line HCP.

Abstract

Residual host cell proteins (HCP) are process related contaminants that can impact the safety and efficacy of drug products. Their detection and clearance is therefore necessary before a drug product can be released. While the ELISA technique has been used extensively for the detection of HCPs, it is a manual technique with precision, sensitivity and time limitations that could be overcome with the use of the Octet® Bio-Layer Interferometry (BLI) platform. Octet® Streptavidin-based biosensors in conjunction with cell line specific polyclonal HCP antibodies can be used to develop a simple method for a more rapid and precise HCP detection assay than ELISA.

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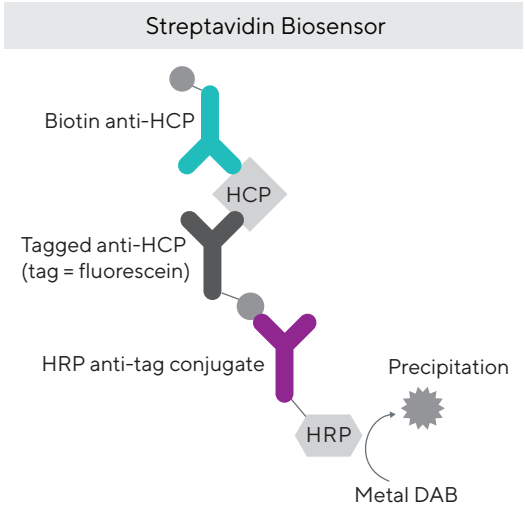
Introduction

Host cell proteins (HCPs) are contaminants found in biopharmaceuticals that originate from the production cell lines of bacterial, yeast or mammalian cell culture. HCPs can reduce drug efficacy and delay or kill promising drug candidates through adverse patient reactions. ELISA is a common analytical method that utilizes a broadly reactive panel of polyclonal antibodies to detect the hundreds of potential Host Cell Proteins (HCPs) contaminating final drug substances. However, there are several inherent problems with ELISA stemming from its reliance on highly manual processing steps that introduce a higher degree of variability in measurement, multiple long, time-consuming incubation steps, and an end-point signal detection relying on colorimetric or fluorescent probes that can provide false positive output.

The Octet® BLI platform provides a superior alternative to ELISA with improved precision in measurements, equivalent or better sensitivity and dynamic range, low manual intervention, rapid assay development enabled by label-free real-time monitoring, and fast time-to-results. The Dip and Read assay method uses parallel processing of samples on disposable biosensors to minimize cross-contamination issues. Generic CHO HCP assays for use in early phases of clinical product development have been validated on the Octet® BLI platform. Process-specific HCP assays displaying full specificity may also be developed once the drug has passed the proof-of-concept stage.

This technical note outlines a protocol for developing and routinely running an assay to detect residual host cell proteins. The protocol may be applied using commercially available generic HCP antibodies, or, process-specific ones. The Octet® Dip and Read assay for HCP proteins involves constructing a sandwich type assay on a Streptavidin (SA) or high precisions Streptavidin (SAX and SAX2) Biosensors on Sartorius' Octet® BLI platform (Figure 1) and takes advantage of the real-time data monitoring capability of Octet® BLI systems to speed up reagent qualification and assay development. The protocol also relies on the highly parallel approach of processing up to 96 samples simultaneously on the Octet® AS instrument offline immobilization station to enable a rapid assay with high sensitivity for detecting HCP contaminants.

Figure 1
Construction of an Assay to Quantitate the Amount of Host Cell Proteins (HCPs) using a Streptavidin (SA) Biosensor on the Octet® BLI Platform.



Reagents Required

- At least 0.75 mg of purified anti-HCP antibody in amine free buffer (i.e., no Tris) at a concentration of 1–1.5 mg/mL (data in this technical note was produced using Cygnus Technologies anti-CHO-HCP antibody, part no. 3G-0016-AF).
- Samples to be analyzed (including positive and negative controls); volume of 200 µL per test
- HCP calibrator of choice; sufficient calibrator to make standards in the range of 0.5–200 ng/mL
- Sucrose (Sigma part no. S0389)
- PD-10 columns (Amersham part no. 17-0851-01) or Zeba spin columns MWCO 7K (Thermo Scientific part no. 89890)
- EZ-Link NHS-PEG_4-Biotin, No-Weigh Format (Thermo Scientific part no. 21329)
- Fluorescein-NHS (Thermo Scientific part no. 46410)
- DMF (Thermo Scientific part no. 20672)
- Metal Enhanced DAB Substrate Kit (Thermo Scientific part no. 34065)
- 1 mL of rabbit HRP-labeled anti-FITC antibody (AbD SeroTec part no. 4510-7864)
- PBS (azide-free)
- 1 bottle of Octet® Sample Diluent (Sartorius part no. 18-1104)
- 1 tray of 96 Streptavidin (SA) Biosensors (Sartorius part no. 18-5019/tray, 18-5020/pack, 18-5021/case)

Equipment Required

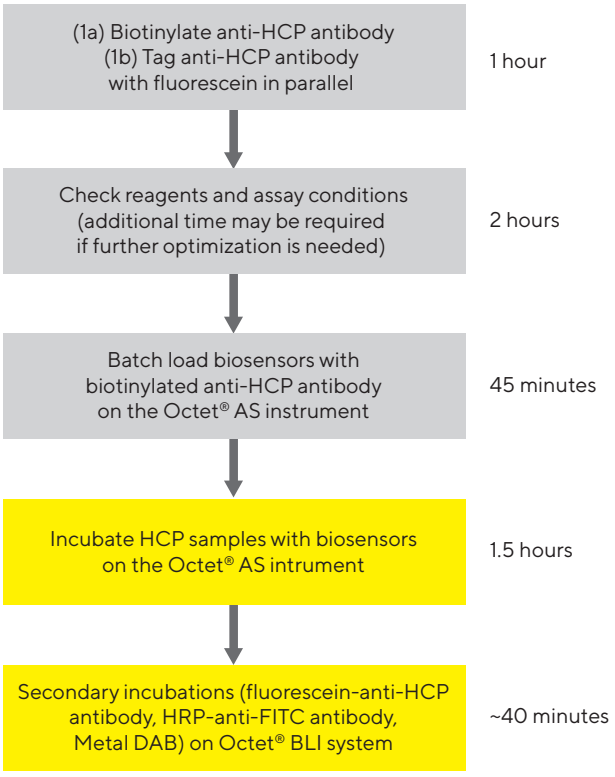
- 96-well microplates (Greiner Bio-One part no. 655209) or any Sartorius approved sample plates
- Centrifuge for Zeba spin columns
- Octet® AS Offline Biosensor Immobilization station
- Octet® R8, RH16 and RH96 BLI systems with software version 11.0 or later

HCP Assay Development Workflow

One aliquot of the anti-HCP antibody must be biotinylated to allow for easy immobilization onto the biosensor. A second aliquot of the anti-HCP antibody must be tagged using a non-biotin based tag. A fluorescein tag is recommended as it is readily available, easy to use, and has commercially available antibodies already conjugated to HRP for detection. Alternatively, HRP conjugation can be directly performed on the anti-HCP antibody. The two approaches to making the secondary antibody may affect assay performance. See Harlow and Lane, Antibodies for more information on this bio-conjugation protocol. Both the biotinylation and the tagging of the antibody can be performed on two separate aliquots of the anti-HCP antibody in parallel.

Figure 2

Flowchart Shows the Steps for Developing and Running an Octet® HCP Assay.



Note. Flowchart shows the steps for **developing** and **running** an Octet® HCP assay. The steps in gray boxes need to be performed to prepare the reagents and biosensors for use in the HCP assay. The boxes in yellow represent steps that are performed to routinely run the HCP assay.

Biotinylating the Anti-HCP Antibody

1. If the antibody is in an amine-based buffer (e.g., Tris), buffer exchange into PBS using Zeba spin column or PD-10, according to the column manufacturer's instructions.
2. Dilute or concentrate the antibody to a stock concentration of 1–1.5 mg/mL.
3. Calculate the moles of antibody to be biotinylated. It is recommended to biotinylate at least 0.5 mg of antibody in order to coat up to 10 trays of 96 biosensors each.
4. Calculate the amount of 2 mM biotin reagent needed to obtain an MCR of 3 (3 moles of biotin for 1 mole of antibody).
5. Obtain 1 tube of EZ-Link NHS-PEG_4-Biotin, No-Weigh Format.
6. Prepare a 20 mM stock solution by adding 170 µL of DI water.
7. Dilute the 20 mM stock solution 1:10 with DI water to get a 2 mM working solution.

8. Add the calculated volume of biotin-PEG_4-NHS to the antibody solution slowly while gently vortexing (dropwise).
9. Incubate for 30 minutes at room temperature (20–25°C).
10. During incubation, prepare PD-10 or Zeba spin columns for buffer exchange according to the column manufacturer's instructions.
11. Remove excess biotin using PD-10 columns or Zeba spin columns according to the column manufacturer's instructions.
12. The biotinylated antibody should be stored at 4°C. Typically, biotinylated antibodies are stable at 4°C for at least 1 month. However, stability is antibody dependent and should be monitored.

Tagging the Anti-HCP Antibody

1. If the antibody is in an amine-based buffer (e.g., Tris), buffer exchange into PBS using Zeba spin column or PD-10 according to the column manufacturer's instructions.
2. Dilute or concentrate the antibody to a stock concentration of 1–1.5 mg/mL.
3. Calculate the moles of antibody to be tagged with fluorescein-NHS. It is recommended to tag at least 0.25 mg of antibody for ease of handling. This amount of tagged antibody is enough to run up to 250 assays.
4. Calculate the amount of 21 mM (10 mg/mL) fluorescein-NHS reagent needed to obtain an MCR of 15 (15 moles of fluorescein-NHS for 1 mole of antibody)
5. Prepare a 21 mM solution of fluorescein-NHS in DMF. Vortex to mix thoroughly.
6. Add the calculated volume of fluorescein-NHS to the antibody solution slowly while gently vortexing (dropwise) and incubate for 30 minutes at room temperature (20–25°C).
7. During incubation, prepare PD-10 columns or Zeba spin columns for buffer exchange according to the column manufacturer's instructions.
8. Remove excess fluorescein-NHS using PD-10 columns or Zeba spin columns according to the column manufacturer's instructions.
9. Fluorescein-tagged antibody should be stored at 4°C protected from light. Typically, tagged antibodies are stable at 4°C for at least 1 month. However, stability is antibody dependent and should be monitored.

Assay Development and Optimization

Check the activity of the biotinylated and fluorescein-tagged antibodies by running a step-by-step kinetics assay with the reagents. Optimize the biotinylated antibody immobilization step as well as other assay conditions. The experiments described in this section may or may not be sufficient to optimize the assay to your particular requirements. For assay optimization beyond that mentioned in this section, re-run with appropriate reagents or condition modifications.

Note: Sample Diluent used in this assay should be azide free. The presence of azide can inhibit the activity of the HRP enzyme.

Set Up the Assay

1. Prepare at least 1.7 mL each of the following reagents:
   a. 12.5 µg/mL of biotin-anti-HCP antibody in Sample Diluent
   b. 5 µg/mL of fluorescein-anti-HCP antibody in Sample Diluent
   c. 1:1000 dilution of the HRP-anti-FITC antibody in Sample Diluent
   d. 1:10 dilution of the DAB-metal substrate in Stable Peroxide Buffer
2. Prepare at least 220 µL each of HCP standards in Sample Diluent at the following recommended concentrations: 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 5 ng/mL, 2.5 ng/mL, 0.5 ng/mL and 0 ng/mL.
3. Prepare the sample plate as shown in Figure 3 in a black, flat-bottomed 96-well microplate. Fill each well with 200 µL of the reagent specified.
4. Set up the kinetic assay steps shown in Table 1 for 8 biosensors on the Octet® BLI system. Total assay time is roughly 1.5 hours.
5. Prepare a hydration plate by pipetting 200 µL of Sample Diluent into 1 column (8 wells) of a black, flat-bottom 96-well microplate.
6. Place the hydration plate under 8 Streptavidin Biosensors to be used in the assay and incubate for at least 10 minutes.
7. Place the hydrated biosensors (with hydration plate) and sample plate on the sample plate holder of the Octet® BLI system.
8. In the Data Acquisition component of Octet® software, go to the Run Experiment tab and enter a value of 600 seconds in the Delayed experiment start field in order to give the sample plate at least 10 minutes inside the Octet® BLI system to equilibrate to the assay temperature of 30°C.
9. Click **GO** to run the assay.

Figure 3

Sample Microplate Map to Check for Reagent Activity.

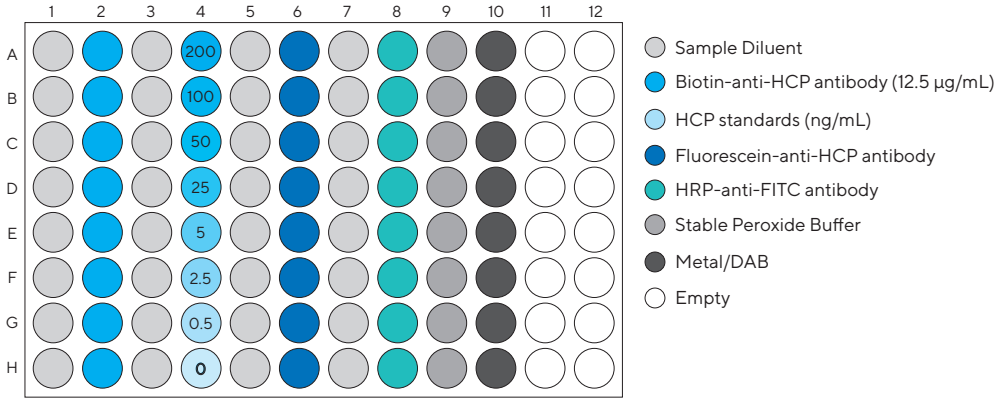
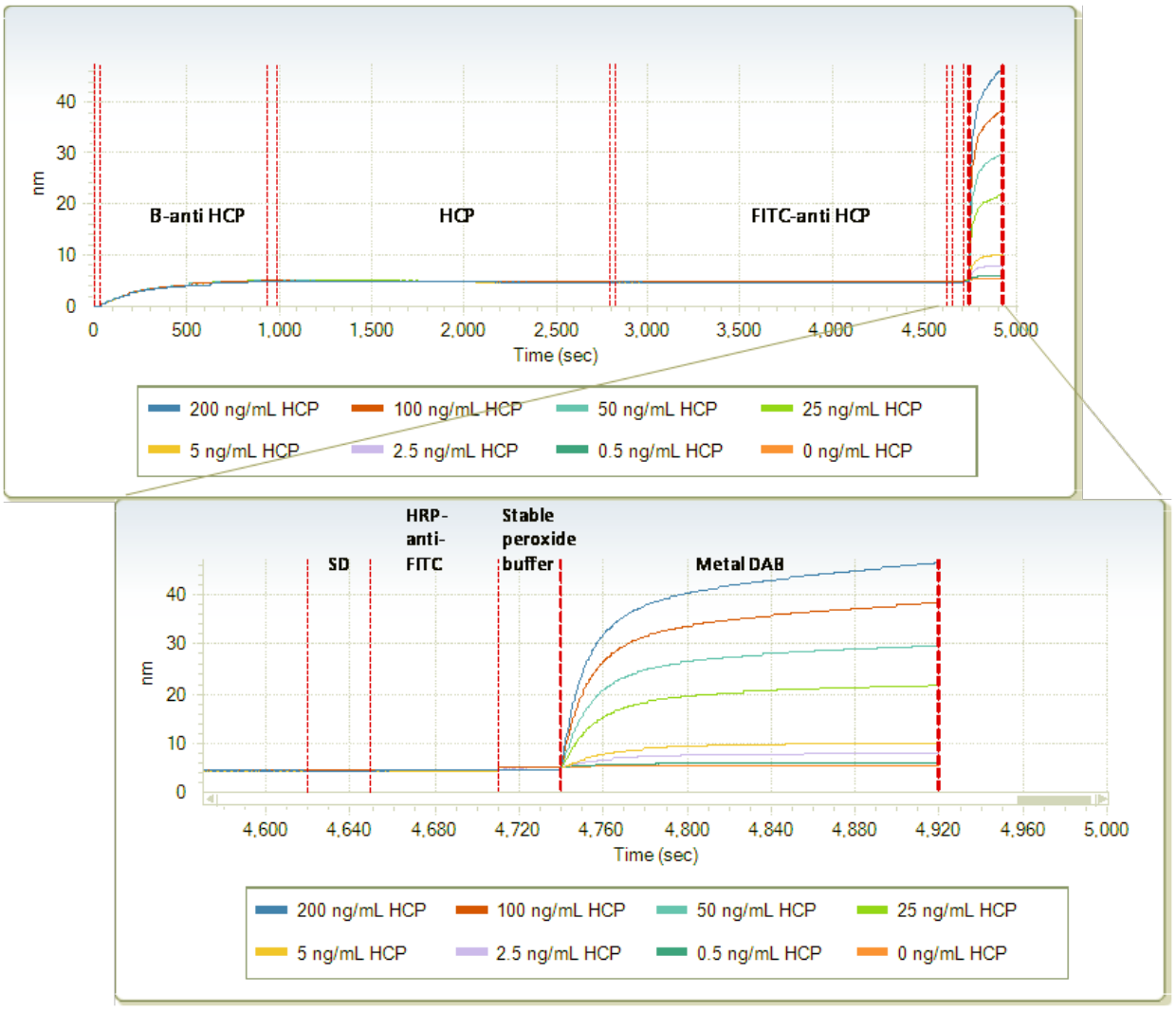


Table 1

Assay Steps, Sample Plate Columns and Step Times ad Shake Speeds for the Experiment That Checks for Reagent Activity.

Step No.	Sample Plate Column No.	Step Name	Time (sec)	Shake Speed (rpm)	Step Type
1	1	Baseline	30	1000	Baseline
2	2	Biotin-anti-HCP	900	1000	Load
3	3	Baseline 2	60	1000	Baseline
4	4	Sample incubation	1800	1000	Custom
5	5	Baseline 3	30	1000	Baseline
6	6	Fluorescein-anti-HCP	1800	1000	Custom
7	7	Baseline 4	30	1000	Baseline
8	8	HRP-Anti-FITC	60	1000	Custom
9	9	Rinse	30	200	Baseline
10	10	DAB-metal	180	200	Association

Figure 4
Sample Real-time Data from the HCP Assay.



Assess Reagent Activity and Assay Conditions

Load the completed experiment from the above run into Octet® Analysis Studio Software. The assay data should look similar to Figure 4.

Align the loading of the biotin-anti-HCP antibody to visualize the immobilization onto the sensor (Figure 5).

The following minimal attributes should be met by the loading step signals for a good assay:

- The loading step should show an nm signal shift of at least 2 nm.
- The signals should come to a plateau prior to the end of the step, indicating that surface immobilization has stabilized.
- The signal should be stable when going into the baseline step following the loading step.
- If any of these things are not true, see the section on Troubleshooting in Appendix I.
- If all of the above is true, then the loading times and concentrations of the biotin-anti-HCP antibody are appropriate for use in the batch loading on biosensors using the Octet® AS instrument.
- Align the signals for the metal DAB step to visualize the dose response of the assay (Figure 6).

The signals obtained in this step should have several key attributes:

- The nm signal shifts should show dependence on the HCP concentration in the samples.
- The lowest concentration should be distinctly higher in signal than the blank (0 ng/mL HCP).
- The signals for the highest concentration samples should be distinguishable from each other.
- If any of these things are not true, see the section on Troubleshooting in Appendix I.
- Click **Quantitate the Selected Step** and produce a calibration curve based on the R equilibrium binding rate equation (example in Figure 7).

The resulting calibration curve provides data for the dynamic range of the assay. The assay dynamic range can be tuned by, among other factors, changing the incubation time of the HCP samples with biosensors. Longer incubation time will shift the dynamic range to lower concentrations. Shorter incubation time will shift the dynamic range to higher concentrations.

If the dynamic range and curve separation are acceptable, then the sample incubation and fluorescein-anti-HCP antibody incubation times can be considered optimal for the HCP assay.

Figure 5
Aligned Loading Step from the HCP Assay.

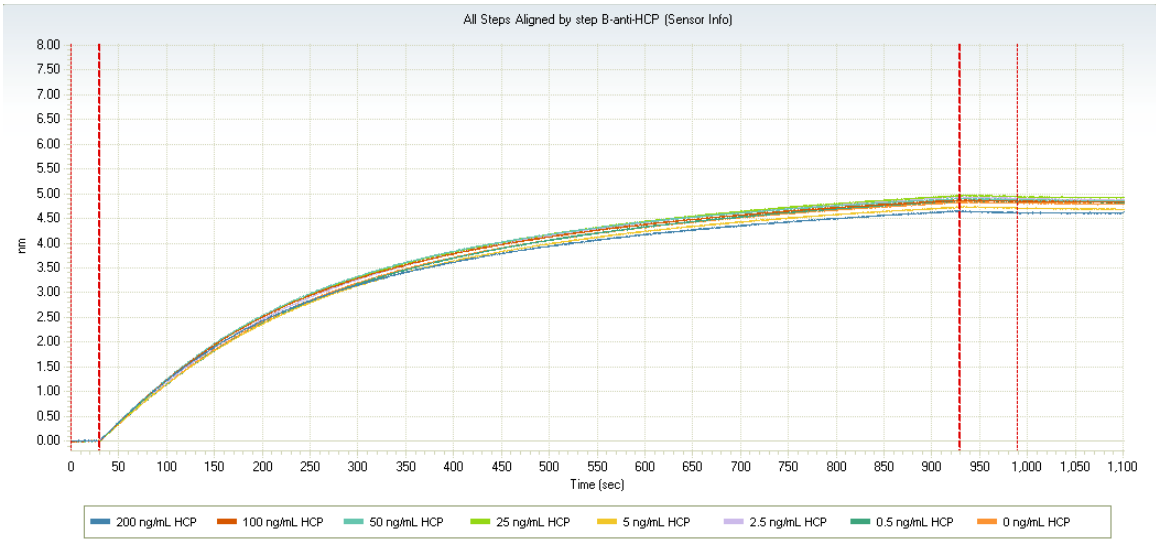


Figure 6
Dose Response for the Metal-DAB Incubation Step in the HCP Assay.

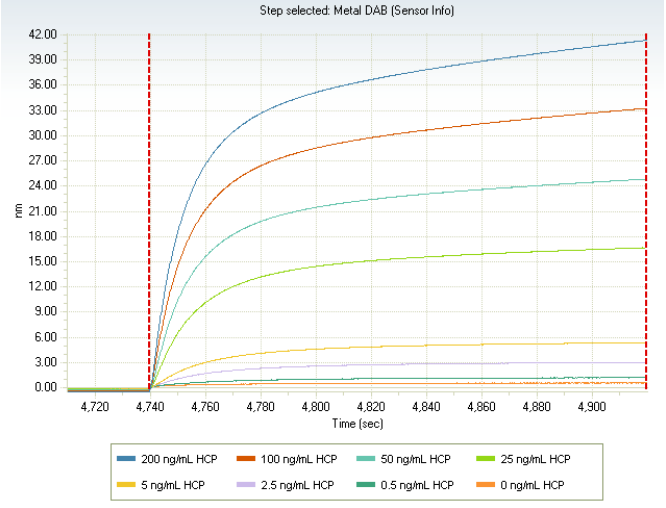
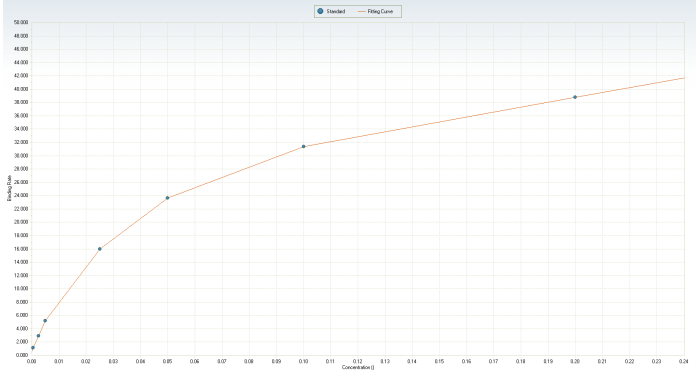


Figure 7
Example Calibration Curve for the HCP Assay.



Batch-Loading Biotinylated Anti-HCP Antibody on Biosensors

For an assay in which precision is important, it is worth spending the time to batch-load biosensors with the biotinylated anti-HCP antibody. The antibody-loaded biosensors can be preserved using a 15% (w/v) solution of sucrose, dried and stored in the biosensor pouches at room temperature until they are needed. Once antibody-loaded biosensors have been prepared, the HCP assay can be completed in ~2 hours.

The following protocol assumes use of a Octet® AS instrument station. Use of a Octet® AS instrument is essential in all offline incubations of biosensors with reagents to obtain optimal performance in the HCP assay.

1. Equilibrate all reagents to room temperature and mix prior to use.
2. Determine the number of Streptavidin Biosensors to be coated and preserved. Place the exact number of biosensors to be loaded with antibody in the biosensor tray.
3. Prepare the hydration plate by pipetting 200 µL of Sample Diluent into wells of a polypropylene 96-well microplate. The number of wells filled and their location should match the location of biosensors in the biosensor tray. Keep the microplate covered until use.
4. Prepare the loading plate:
   a. Dilute the biotin-anti-HCP antibody solution to 12.5 µg/mL in Sample Diluent or, to the concentration value determined as optimal in the biosensor loading optimization experiment. A 12.5 µg/mL solution can typically be used up to 10 times to coat successive trays of Streptavidin Biosensors. Total time on the Octet® AS instrument should be limited to less than 3 hours in order to limit the loss of liquid by evaporation.
   b. Pipette 200 µL of the biotin anti-HCP antibody solution into wells on a 96-well plate. The number of wells filled and their location should match the location of biosensors in the biosensor tray. Keep the microplate covered until use.
5. Prepare the rinse plate by pipetting 200 µL of Sample Diluent into wells of a polypropylene 96-well microplate. The number of wells filled and their location should match the location of biosensors in the biosensor tray. Keep the microplate covered until use.
6. Prepare the preservation plate:
   a. Make a 15% (w/v) solution of sucrose in nanopure water.
   b. Pipette 200 µL of the sucrose solution into a separate 96-well plate. Keep the microplate covered until use.
7. Using the Octet® AS instrument, coat the Streptavidin Biosensors according to the following protocol at 30°C.
   a. Hydrate the Streptavidin Biosensors in the hydration plate for 10 minutes without shaking.
   b. Incubate the Streptavidin Biosensors in the loading plate for 15 minutes (or an incubation time determined during biosensor loading optimization) at 1000 rpm shake speed.
8. Rinse the biosensors in the rinse plate for 30–60 seconds at 1000 rpm.
9. Incubate the biosensors in the preservation plate for 1–3 minutes at 1000 rpm.
10. Dry the biosensors in a 37°C oven for 2–5 minutes.
11. Store the dry, preserved biosensors in the tray sealed in the original foil pouch with the provided desiccant.

Note: All of the above incubation steps employ a shake speed of 1000 rpm. This high shake speed provides fast and efficient loading of reagents on biosensors. However, if required by your assay, the shake speed can be modified.

HCP Assay Protocol

Once the biosensors have been loaded with biotinylated anti-HCP antibody and the biosensor loading optimization has been done, the HCP assay is performed according to the steps shown in Figure 8.

For the longer incubation steps (incubation of samples and fluorescein-tagged anti-HCP antibody), up to 96 samples can be processed in parallel using the Octet® AS instrument. Once the biosensors and samples have been processed, then a short set of HRP-anti-FITC antibody and metal-DAB incubation steps are run in the Octet® BLI system.

Sample Diluent used in this assay should be azide free. The presence of azide can inhibit the activity of the HRP enzyme.

Figure 8
HCP Assay Steps Outlined in a Flow Chart.

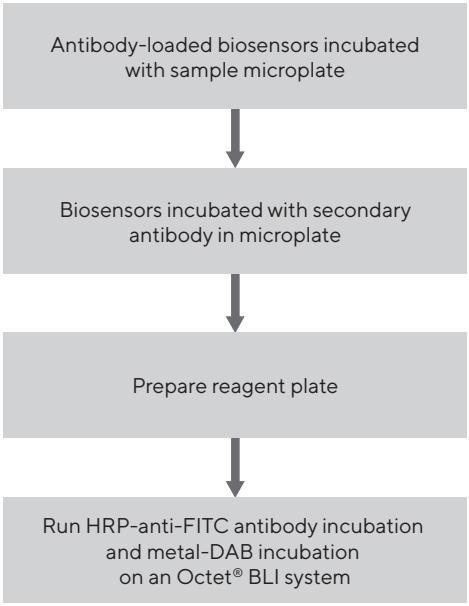
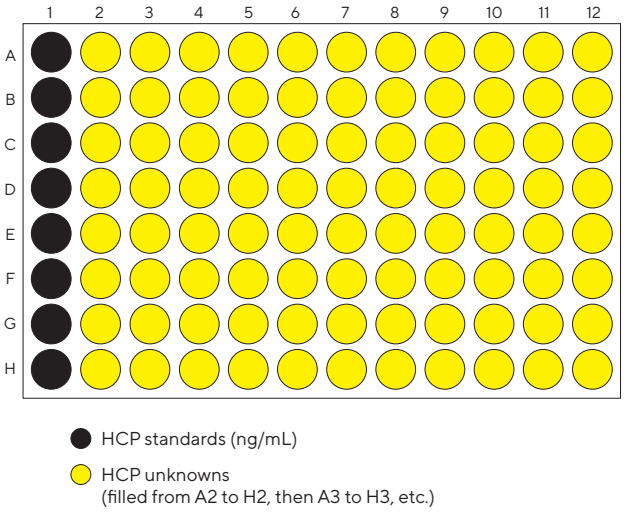


Figure 9
Sample Plate for Incubation with Biosensors on the Octet® AS Instrument.



Process Samples on the Octet® AS Instrument

1. Equilibrate the samples, reagents and buffers to room temperature and mix thoroughly prior to use.
2. Prepare the sample plate (example plate shown in Figure 9):
   a. Prepare at least 220 µL of each concentration of HCP calibration standards in sample diluent. Use the range determined in the Biosensor Loading Optimization experiment.
   b. Pipette 200 µL of each standard into wells starting at A1 and proceeding to H1.
   c. Pipette 200 µL of each HCP unknown sample into wells in the remainder of the microplate.
3. Prepare the sensor tray by moving antibody loaded biosensors into an empty tray. Biosensor locations should correspond to filled wells in the sample plate (1 sensor for each filled well in the sample plate).
4. Prepare a hydration plate by pipetting 200 µL of Sample Diluent into each well of a second polypropylene 96-well plate. Well locations filled with Sample Diluent in the hydration plate should correspond to biosensor locations in biosensor tray.
5. Hydrate biosensors for a minimum of 10 minutes in the Hydration Plate without shaking.
6. Using Octet® AS instrument, incubate hydrated biosensors in the Sample Plate at 1000 rpm, at 30°C for 30 minutes or using conditions found optimal in the **Assess Reagent Activity and Assay Conditions** experiments.
7. During this incubation, prepare the secondary antibody plate.
   a. Prepare a 5 µg/mL solution of fluorescein-anti-HCP antibody in Sample Diluent. Prepare a minimum of 220 µL for each biosensor to be processed.
   b. Pipette 200 µL into each well of a new 96-well microplate. Filled wells should correspond to biosensor location and number in the biosensor tray.
8. After 30 minutes, replace the sample plate with the secondary antibody plate. Incubate on the Octet® AS instrument at 1000 rpm and 30°C for 30 minutes or the incubation time determined in the biosensor loading optimization experiment.
9. During this incubation, prepare the reagent plate for the Octet® assay. Reagent quantities given below are for 8-channel assays on Octet® R8 BLI systems and the 8-channel read head on the Octet® RH16 BLI system (Figure 10). Reagent quantities in parenthesis are for the 16-channel read head on the Octet® RH16 BLI systems (Figure 11).
   a. Prepare at least 1.7 mL (3.4 mL) each of
      - 1:1000 dilution of the HRP-anti-FITC antibody in Sample Diluent.
      - 1:10 dilution of metal-DAB in Stable Peroxide Buffer.
   b. In a black, flat-bottomed 96-well microplate, pipette 200 µL of each reagent specified in the plate map below into each well. Each reagent needs to be filled into all wells of 1 column (2 columns) of the Reagent Plate.

Figure 10
Reagent Plate for 8-channel Assays (Octet® R8 and RH16 BLI Systems).

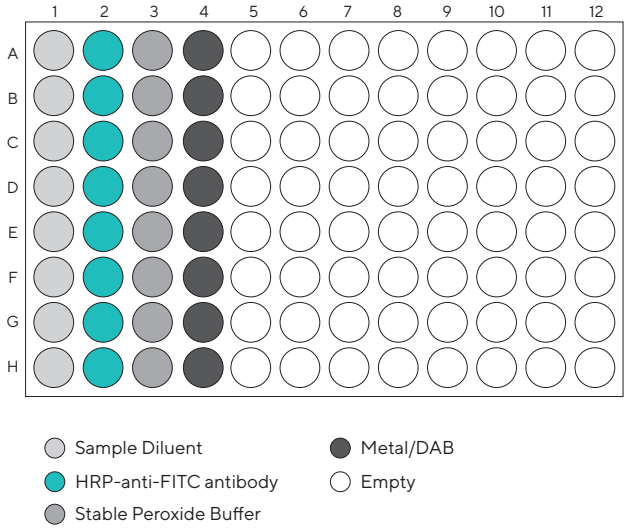
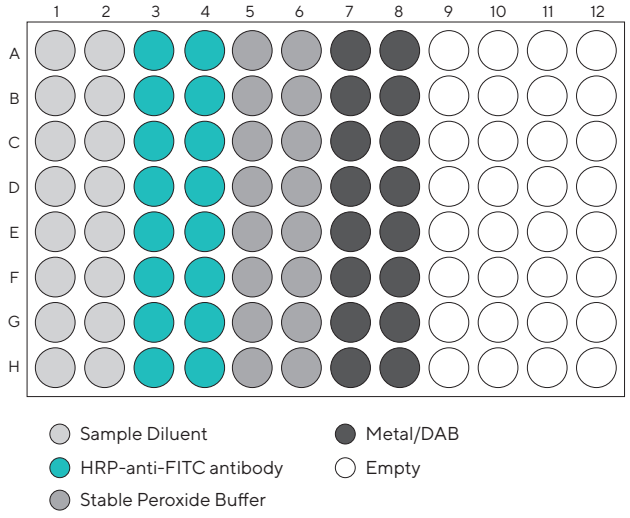


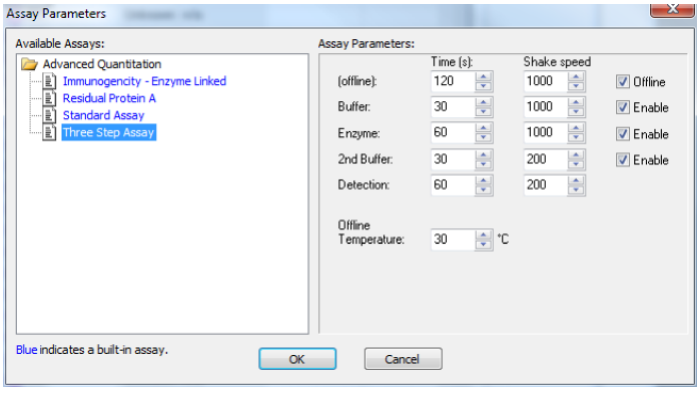
Figure 11
Reagent Plate for 16-channel Assays (Octet® RH16 BLI System only).



Run Detection Steps on the Octet® BLI System

1. In a black, flat-bottom 96-well microplate, pipette 200 µL of Sample Diluent into each well corresponding to the position of the biosensors in the biosensor tray. Place the microplate in the sensor deck of the Octet® BLI system.
2. After Octet® AS instrument incubation of biosensors with the fluorescein-anti-HCP antibody is complete, place the biosensor tray over the newly pipetted Sample Diluent plate in the Octet® BLI system.
3. Place the reagent plate in the Octet® BLI system.
   - In the Octet® R8 BLI system, place the reagent plate on the sample plate station.
   - In the Octet® RH16 BLI system, place the reagent plate in the reagent plate station.
4. Open the Octet® BLI Discovery Software and choose the Advanced Quantitation option in the Experiment wizard. In the Assay Settings window, click **Modify** to set up the assay parameters as shown in Figure 12.
5. Click **OK**.
6. In the Octet® RH16 BLI system, define the Sample Plate layout to correspond to the sample plate layout used in Octet® AS instrument incubation.
7. In the Octet® RH16 and RH96 BLI systems, define the reagent plate layout to correspond to the reagent plate created. In the Octet® R8 BLI system, the sample plate layout is defined to correspond to the reagent plate created.
   a. Sample diluent = (B) Buffer
   b. HRP-anti-FITC antibody = (E) Enzyme
   c. Stable Peroxide Buffer = (2) 2nd Buffer
   d. Metal-DAB = (D) Detection
8. Enter Sample and Sensor information in the Plate Definition tab and the Sensor Assignment tab as desired.

Figure 12
Assay Settings to Use for Advanced Quantitation to Run the HCP Detection Assay.



9. In the Run Experiment tab, enter a delay time of 600 seconds in order to give the reagent plate at least 10 minutes inside the Octet® BLI system to equilibrate to assay temperature.
10. Enter location and file name for saving the data.
11. Click **GO** to run the assay.

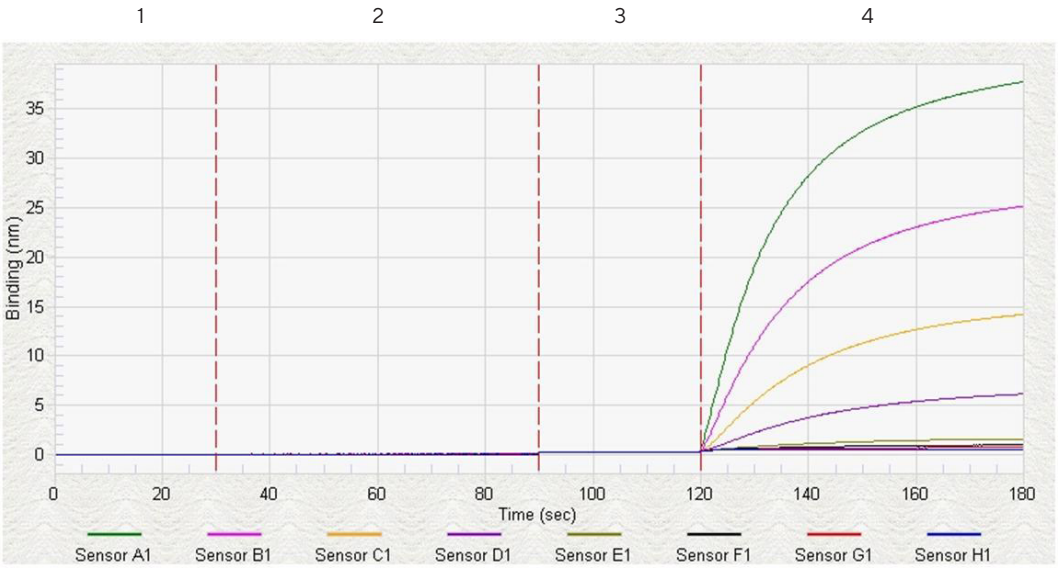
Analyze Data

1. In the Octet® Analysis Studio Software, load the data folder to be analyzed.
2. Select the reference well and do reference subtraction if needed.
3. Group and Concentration information can be modified in the table if needed.
4. In the Results tab:
   a. Select the desired standard curve. If no standards were included in the experiment, then the output will be binding rates only.
   b. Select **R equilibrium** as the binding rate equation. This equation will fit the binding curve generated during the experiment and calculate a response at equilibrium as the output signal.
   c. Click on **Calculate Binding Rate**. Results will be displayed automatically in the table.
   d. Click the **Save Report** button or select **File > Save Report** to generate a Microsoft® Excel report File.

Example Data from a Routine Asssay

The data shown was generated using the Cygnus anti-CHO-HCP antibody (Cygnus part no. 3G-0016-AF). The antibody was loaded on Streptavidin Biosensors using the protocol outlined in this technical note. The CHO-HCP standards at 200, 75, 25, 8, 2, 1, and 0.5 ng/mL in Sample Diluent were made and run in triplicate. Three unknowns (Sample 1, 2 and 3) were run in 8-fold replicates to assess assay precision.

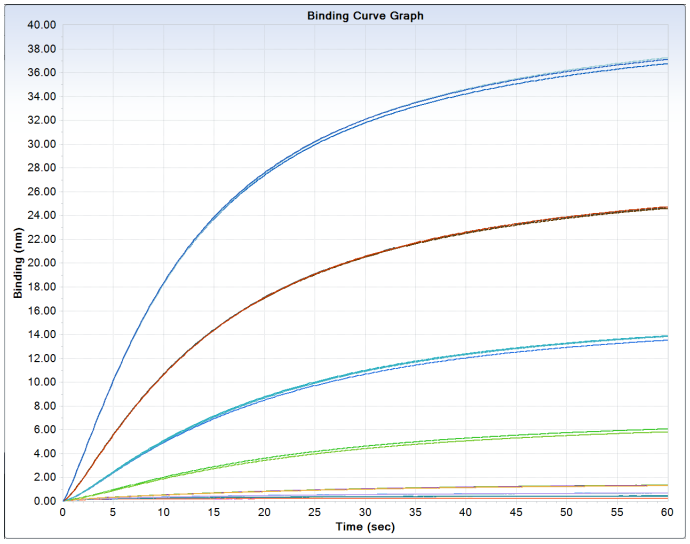
Figure 13
Real-time Data From a CHO-HCP Assay.



Note. Biosensor processing with samples and binding with the fluorescein-tagged anti-CHO-HCP antibody were performed using the Octet® AS instrument. Data shown is from the detection assay run on an Octet® R8 BLI system. The steps shown include 1) baseline in Sample Diluent, 2) binding of HRP-anti-FITC antibody, 3) Stable Peroxide Buffer equilibration and 4) detection of signal using metal-DAB substrate. The assay was run according to the procedure outlined in this technical note.

Figure 14

Example Data from the Analysis of the Metal-DAB Detection Step in the Assay.

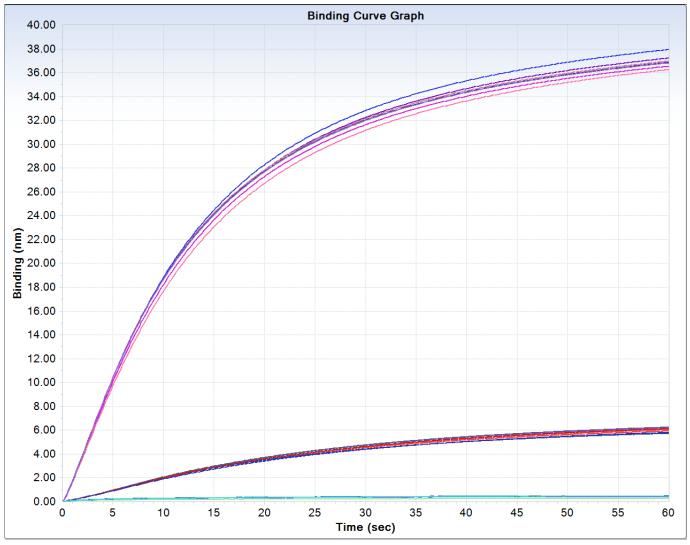


Expected Concentration (ng/mL)	Calculated Concentration (ng/mL)	Recovery	CV
200.0	201.0	101%	2.1%
75.0	73.9	99%	0.7%
25.0	25.5	102%	2.3%
8.0	8.08	101%	2.8%
2.0	1.72	86%	1.8%
1.0	0.88	88%	1.7%
0.5	0.50	100%	3.3%

Note. The data shows a dose response for the calibration standards (in triplicate). The calculated concentrations and %CV values resulting from the analysis of the data are shown in the accompanying table.

Figure 15

The Graph Shows Data for 3 Samples, Each Replicated 8-fold.



Sample	Calculated Concentration (ng/mL)	CV
Sample 1	36.5	4.1%
Sample 2	6.07	4.0%
Sample 3	0.43	5.1%

Note. The calculated concentrations and %CV are shown in the accompanying table. The concentrations were calculated using the calibration data shown in Figure 14.

Appendix I: Troubleshooting

Issue	Possible Reasons	Suggested Solutions
Biotin antibody loading signal is < 2 nm	Free biotin may still be present in biotin-antibody.	Re-purify biotinylated protein using Zeba spin column or PD-10.
	Amine contaminant in antibody solution.	Buffer exchange into PBS prior to re-biotinylating (use dialysis, PD-10 or Zeba spin columns)
	Biotin NHS reagent is hydrolyzed.	Use new aliquot of biotin NHS reagent. Store reagent protected from moisture at 4°C or as directed by manufacturer to prevent hydrolysis.
Biotin-antibody binding does not come to a plateau prior to the end of the loading step	Biotin-antibody active concentration is lower than expected.	Increase concentration of biotin-antibody. OR Increase loading time until plateau is reached.
Biotin-antibody binding is not stable after the loading step	Biotin-antibody has aggregated and aggregates are falling apart after loading.	Decrease the MCR of biotinylation step to avoid aggregation due to over biotinylation.
No or low signal from HCP standards	Concentration of standards below assay range.	Increase concentration of HCP standard and check for binding.
	HCP antibody is not active.	Check activity of HCP antibody.
	Peroxide buffer or metal-DAB is no longer active.	Check for activity by combining peroxide buffer, metal-DAB with 1 µL of HRP-anti-FITC antibody. A black precipitate should appear if all are active.
	Sample incubation times are not long enough.	Increase sample incubation time up to 1 hour.
	Active HRP concentration is not high enough.	Increase HRP-anti-FITC antibody concentration.
	Background is too high.	Dilute metal-DAB solution by 10 fold.
Highest concentrations of HCP in standard curve are not distinguishable from each other	High concentrations are out of the dynamic range.	If the high concentrations are important, tune the dynamic range of the assay by shortening the sample incubation time.

Germany
Sartorius Lab Instruments GmbH & Co. KG
Otto-Brenner-Strasse 20
37079 Goettingen
Phone +49 551 308 0

USA
Sartorius Corporation
565 Johnson Avenue
Bohemia, NY 11716
Phone +1 888 OCTET 75
Or +1 650 322 1360

For further contacts, visit
www.sartorius.com/octet-support

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