Protocol

Quantification of Host Cell Proteins using Mass Spectrometry - Analytical Method Validation - Non GMP

PI Alphalyse	**Reviewer Alphalyse**
_____	_____
Date / Name	Date / Name
Client QA	**Client PAD**
_____	_____
Date / Name	Date / Name

Effective date: Date-Month-Year

Document Owner
NOT GMP

1. Scope

The present scope is Non-GMP Method Validation to support use of the method as a release assay in testing of Clinical Trial Material (CTM). Design of the Non-GMP validation protocol addresses the validation parameters in the ICH Guideline; Validation of analytical procedures Q2(R1), for intended use in quantitative testing of impurity content.

The test is an LC-MS/MS method to identify and quantify Host Cell Proteins (HCP) from the *"Insert Producer cell name"* host cell of Drug Substance samples.
Alphalyse has developed a quantitative LC-MS/MS method for identification and quantification of HCPs in Sponsor samples and analyzed one to two in-process steps of the representative DS "*Insert DS sample name here*" (see development report xxxxx).

Sponsor intends to use the Non-GMP validated method for analysis and release of batches for clinical use.

Table of Content

2. Summary of Test Parameters for the Non-GMP validation

Non-GMP Method Validation is done according to the ICH Guideline; Validation of analytical procedures Q2(R1) to test the performance of the method for its suitability as a quantitative impurity assay to test HCP clearance. The parameters validated include accuracy, LLOQ and ULOQ, linearity, repeatability, precision, range, and specificity.

The design of the qualification protocol addresses ICH Q2 parameters as follows:

Accuracy is determined by spiking seven standard proteins (Table 2) into DS at 8 different levels: 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 500 ng/mL, 2,000 ng/mL, 5,000 ng/mL, and 15,000 ng/mL. The response factor of beta-lactoglobulin (LACB) at 2000 ng/mL is used to determine the accuracy of the standard proteins. Accuracy is confirmed if the recovery of the seven standard proteins is acceptable (50-200% in 4 out of the 7 proteins, not necessarily to be the same 4 protein at all spike-in levels). Spike-in levels below LLOQ and above ULOQ are excluded.

LLOQ and ULOQ is determined as the lowest and highest level in which the recovery of the seven standard proteins is acceptable (50-200% in 4 out of the 7 proteins). Precision at LLOQ and ULOQ is demonstrated by Protein1 and Protein3 (Table 4).

Linearity is obtained from the accuracy experiment data, using the data for beta-lactoglobulin (LACB, Table 2). The coefficient of determination (R^2) should be acceptable (≥0.95). Spike-in levels that are below LLOQ and above ULOQ are excluded in the linearity test.

Repeatability is demonstrated within a single run using one analyst (Intermediate precision - Day 1). Repeatability is determined for three endogenous proteins found in DS (Table 4) as well as for the total HCP content. The level of the selected proteins should be around LLOQ (low level), 500 ng/mL (medium level) and upper limit of quantification (ULOQ). Coefficient of variation (CV) of the protein concentration should be ≤25%.

Intermediate precision (between day repeatability) is determined by performing a 3 X 3 matrix: DS will be run on different days (Day 1, Day 2, and Day 3), with two different sample preparation analysts (Analyst 1 and Analyst 2). Three replicates are performed each day giving a total of 9 determinations. Intermediate precision is assessed for the same three proteins as for repeatability (Table 4) as well as for the total HCP concentration in each of the preparations. Coefficient of variation (CV) of the protein concentration should be ≤25%.

The Range is defined as the concentration interval for which it has been demonstrated that the present analytical procedure has a suitable level of precision, accuracy, and linearity.

Specificity is confirmed by showing that the concentration of endogenous proteins in the DS sample (same three endogenous proteins as for repeatability and intermediate precision, Table 4) is not affected by the presence of spike-in proteins, such as the seven spike-in standard proteins at 2000 ng/mL. The concentration of the three endogenous proteins is compared in spiked and un-spiked DS samples. A t-test is used to determine if the concentration of the three endogenous proteins and the total HCP concentration is significantly different between the spiked and un-spiked DS samples.

3. Analytical Method

The qualification is performed using the Analytical Method: Document xxx Drug Sample Preparation for Non-GMP Method Validation Study xxxxx.

3.1 Reference sample, standards, and normalization protein

A DS reference sample will be used for the Non-GMP validation study (Table 1). A protein standard mixture of seven intact proteins (Table 2) will be spiked into the samples to be used as internal protein standards. Additionally, the standard proteins will be spiked-in at different concentrations for the linearity experiment, accuracy, LLOQ and ULOQ. The DS reference samples for linearity, accuracy, LLOQ and ULOQ are also spiked with a normalization protein (Table 3) at 2500 ng/mL. The acquired data for the samples containing the normalization protein, will be normalized prior to data analysis. The normalization protein is added to be able to compare responses across individual samples for the linearity experiment. The normalization protein is not used during routine analysis. For routine analysis the response factor for LACB spiked-in to each sample is used.

Table 1. Reference DS sample

Lot no.	Sample name
xxx	xxx

Table 2. Standard Proteins

Proteins	Mass (Da)	Protein Names
P68082|MYG_HORSE	17083	Myoglobin
G5E5H7|LACB_BOVIN	19883	Beta-lactoglobulin
P00698|LYSC_CHICK	16239	Lysozyme C
P01966|HBA_BOVIN	15184	Hemoglobin subunit alpha
P02070|HBB_BOVIN	15954	Hemoglobin subunit beta
P00709|LALBA_HUMAN	16225	Alpha-lactalbumin
P02787|TRFE_HUMAN	77064	Serotransferrin

Table 3. Normalization protein used for linearity

Protein	Mass (Da)
NISTmAb, Humanized IgG1$_k$ Monoclonal Antibody, Heavy chain	49596
NISTmAb, Humanized IgG1$_k$ Monoclonal Antibody, Light chain	23123

3.2 Accuracy

To determine accuracy, seven standard proteins will be used. The DS sample (Table 1) is spiked with 15,000 ng/mL of each standard protein and diluted in an un-spiked sample (0 ng/mL) to 5,000, 2,000, 500, 100, 50, 25, and 10 ng/mL spike-in. The DS sample is also spiked with a normalization protein (2500 ng/mL, Table 3), and the acquired data will be normalized prior to data analysis. The response factor of LACB at 2000 ng/mL is used to determine the concentration of the seven standard proteins at each spike-in level. The accuracy of the standard proteins is examined. Accuracy is obtained if 4 of the 7 standard proteins are recovered between 50-200 % at concentrations above LLOQ and below ULOQ. It does not have to be the same 4 proteins at each spike-in level.

Recovery is defined as:

$$\%Recovery = \frac{Conc(observed)}{Conc(spiked)} * 100$$

3.3 Lower and upper limit of quantification (LLOQ and ULOQ)

To determine LLOQ and ULOQ, seven standard proteins will be used. The samples prepared for determining accuracy (Section 3.2) will be reused for determining LLOQ and ULOQ. The DS sample (Table 1) is spiked with 15,000 ng/mL of each standard protein and diluted in an un-spiked sample (0 ng/mL) to 5,000, 2,000, 500, 100, 50, 25, and 10 ng/mL spike-in. The DS sample is also spiked with a normalization protein (2500 ng/mL, Table 3), and the acquired data will be normalized prior to data analysis. The response factor of LACB at 2000 ng/mL is used to determine the concentration of the seven standard proteins at all concentrations. The accuracy of the standard proteins is examined. LLOQ and ULOQ is determined as the lowest and highest spike-in level at which at least 4 of the 7 standard proteins show recovery within 50-200%.

Recovery is defined as:

$$\%Recovery = \frac{Conc(observed)}{Conc(spiked)} * 100$$

3.4 Linearity and Calibration curve

To determine linearity the DS sample will be spiked with known concentrations of the standard proteins (Table 2) from 15,000 ng/mL down to 10 ng/mL. The DS sample (Table 1) is spiked with 15,000 ng/mL of each standard protein and diluted in an un-spiked sample (0 ng/mL) giving the following concentrations: 15,000, 5,000, 2,000, 500, 100, 50, 25, and 10 ng/mL. The samples

prepared for determining accuracy, LLOQ and ULOQ (Section 3.2 and 3.3) will be reused for determining linearity. The DS sample is also spiked with a normalization protein (2500 ng/mL, Table 3), and the data for determination of linearity will be normalized prior to data analysis. Linearity will only be evaluated for LACB. Usually, LACB has a median response or close to median response, which makes it an appropriate choice for the linearity determination. The LACB response factor at 2000 ng/mL will be used for all future Drug Substance samples. Spike-in levels below the determined LLOQ and above ULOQ (Section 3.3) will be excluded. The coefficient of determination (R^2) of the regression line will be examined and should be acceptable (≥0.95). The observed LACB amount (ng/mL) of all spike-in concentrations will be compared by using the slope of the regression line as the response factor and by using the response factor of LACB at 2000 ng/mL.

3.5 Repeatability

Repeatability is demonstrated within a single run using one analyst (Intermediate precision - Day 1). Repeatability is determined for three proteins (Protein1-3) found in DS (Table 4) as well as for the total HCP concentration (summed HCP concentration of HCPs >LLOQ). Protein1-3 should preferably be HCPs. If no host cell proteins are present at the three concentrations (LLOQ, medium level and ULOQ), other endogenous proteins, such as DS proteins, will be used instead to examine the repeatability at LLOQ, medium level and/or ULOQ. If no HCPs are present above LLOQ in the DS sample, repeatability for the total HCP concentration will instead be examined by a summed endogenous phage protein concentration. The coefficient of variation (CV) of the protein concentration should be ≤25%.

Table 4. Selected QC proteins for determination of repeatability, intermediate precision, and specificity

QC Proteins	Concentration level
Protein1*	Around LLOQ
Protein2*	Around 500 ng/mL (medium level)
Protein3*	Around ULOQ

*Protein1-3 will preferably be HCPs, but if a representative HCP is not present in the DS sample at LLOQ, medium level and/or ULOQ another endogenous protein(s) is/are used instead, for instance the DS protein

3.6 Intermediate precision

Intermediate precision (between day repeatability) is determined by performing a 3 X 3 matrix: DS will be run on different days (Day 1, Day 2, Day 3), with two different sample preparation analysts. Three replicates are performed each day giving a total of 9 determinations. Intermediate precision is assessed by comparing the concentration of 3 endogenous proteins found in the DS sample (same as for repeatability, Table 4) and the total HCP concentration (same as repeatability). The CV of protein concentration should be ≤25%.

3.7 Range

To determine the range for the assay, the following parameters are evaluated: accuracy, LLOQ and ULOQ, linearity, repeatability, and intermediate precision. The range will be defined as the concentration interval of which it has been demonstrated that this analytical procedure has a suitable level of accuracy (by the seven spike-in standards), linearity (by one spike-in standard) and precision (by three endogenous proteins and the total HCP concentration).

3.8 Specificity

Specificity will be demonstrated by comparing the concentration of three endogenous proteins (Table 4) and the total HCP concentration (same as repeatability) in spiked and un-spiked samples, showing that the presence of spike-in proteins does not affect the endogenous protein concentration in DS sample. Three DS samples are spiked with standard proteins (2000 ng/mL) (Table 2) and compared with three un-spiked DS samples with regards to the concentration of the three endogenous proteins (around LLOQ, medium level and ULOQ, Table 4) and the total HCP concentration (same as repeatability). A *t*-test (two-tailed, $P>0.05$) will be used to compare the protein concentration of Protein1-3 and the total HCP concentration between the two groups (spiked and un-spiked DS samples).

4. Sample preparation plan

Prior to shipment, the DS sample is inactivated by addition of 20% SDS and 1M DTT followed by heat inactivation by the Sponsor at 95°C for 60 min. The final concentration of the added reagents is 4% SDS and 50 mM DTT. The addition of the inactivation reagents results in a dilution of the original sample, with a dilution factor of 1.33. Example: 0.5 mL SDS and DTT + 1.5 mL DS sample = 2 mL diluted DS sample ready for heat inactivation.
Upon arrival at Alphalyse, DS samples are stored at 2-8°C. At the first day of sample preparation, the needed sample amount is transferred from the original tube for analysis, and the leftover sample is then aliquoted into 1 mL aliquots and stored <-60°C for 6 month and then discarded.

A total number of 15 samples will be prepared and analyzed in triplicates. In the Analytical Method - Document xxx, the sample preparation for all samples in the Non-GMP method validation is described in detail.
The 15 samples needed for the assay Non-GMP validation are:

- 8 samples spiked with seven standard proteins in the following concentrations: 15,000, 5,000, 2,000, 500, 100, 50, 25, and 10 ng/mL. The samples are prepared by serial dilution of a 15,000 ng/mL standard protein stock. A normalization protein at 2500 ng/mL is also spiked in.
- 1 sample (without standard spike-in, but with 2500 ng/mL normalization protein spike-in) that is used as diluent in the preparation of serial dilution of the above 8 standard protein spike-in concentrations.
- 3 samples spiked with 2000 ng/mL standard proteins and w/o normalization protein.
- 3 samples prepared w/o standard protein spike-in and w/o normalization protein.

The eight samples with the standard protein spike-in concentrations from 10 to 15000 ng/mL will be used for determining: Accuracy, LLOQ, ULOQ and Linearity (Section 3.2-3.4)

The three samples spiked with 2000 ng/mL standard proteins are prepared on three different days and will are used for determining repeatability (Day 1, Section 3.5) and intermediate precision (Day 1-3, Section 3.6). Fresh aliquots are used for each of the samples prepared on the three different days.

The three samples spike with 2000 ng/mL standard proteins used for intermediate precision and the three samples that are prepared without standard protein spike-in are used to determine specificity by examining if the presence of other proteins (such as the spike-in standard proteins) will affect the protein concentration of endogenous proteins in the DS sample.

5. Abbreviations and definitions

Term	Definitions
CTM	Clinical Trial Material
DS	Drug Substance
DTT	DL-Dithiothreitol
HCP	Host Cell Protein
LACB	Beta-lactoglobulin
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LLOQ	Lower Limit of Quantification
NIST	National Institute of Standards & Technology
ULOQ	Upper Limit of Quantification
SDS	Sodium Dodecyl Sulfate

6. Change log

Version	Change	Effective date
1.0	New Document	**Date-Month-Year**

Document Change Control

Version / Status	**1.0 / Effective**
Replaced documents / Status	**N/A**
Effective Date	**Date-Month-Year**
Change	
New document	
Rationale for change	
Created according to purpose	
Impact	
No impact identified	