How to validate a qPCR method for transgene copy number determination in recombinant CHO cell lines to be in compliance with GMP (good manufacturing practice) in biopharmaceutical industry?

Okršlar Veronika, Škulj Mihaela, Doležal Jena, Andrej Francky

Biopharmaceuticals, Cell and Molecular Biology
Lek Pharmaceuticals d.d.
Kolodvorska 27, SI-1234 Mengeš

Introduction

In biopharmaceutical industry stringent requirements are in place for the validation of analytical methods used for characterisation of products and cell lines producing them. Genetic characterization of MCB (master cell bank) and WCB (working cell bank) prepared from a selected clone (CHO cell line) chosen for production, is one of the steps covered by GMP in the process of recombinant protein production development. In addition to sequencing, Southern blot and Northern blot analysis, a qPCR method for transgene copy number determination was introduced. For GMP purposes the qPCR based method has to be validated in a proper way according to the guidelines of ICH-International Conference on Harmonisation (1), FDA- Food and Drug Administration (2,3). Particular assays were designed to determine the accuracy, precision (intra-assay, intermediate), specificity, selectivity, linearity, the limit of detection, the limit of quantification, range of quantification and test ruggedness and robustness of the developed method.

Method description

Transgene copy number determination by qPCR
A real-time qPCR method for determination of copy number of the transgene integrated into the genome of Chinese hamster ovary (CHO) cell was established. The absolute quantification approach was applied. Specific amplicons on a single copy endogenous gene of the CHO genome and on a specific transgene inserted in the recombinant plasmid were constructed. Standard curves were prepared from genomic DNA of nontransfected CHO cells and extracted recombinant plasmid DNA. Five serial dilutions were prepared in the range from 100-10000 copies per well (100pg/µl-10ng/µl) for genomic DNA and from 500-5x10^6 copies per well (10pg/µl-1fg/µl) for plasmid DNA. Genomic DNA from the transfected CHO cell line (sample) was also prepared in five serial dilutions in the same range as for the standard curve of genomic DNA of nontransfected CHO cells. Relative standard curves were constructed for each sample and efficiency of amplification was calculated. The final reaction volume was 9 µl and each was prepared in triplicates. TaqMan chemistry was used and analyses were performed on an Applied Biosystems Prism 7900HT.

After analysis results were evaluated. Dilutions where the SD (Standard Deviation) of three replicates was more than 0.3 were not used for standard curve preparation and copy number determination. Samples where efficiencies of amplification differed more than 10% from those of the standards were reanalysed. The copy number was determined as a ratio between transgene and endogenous gene copy number in the reaction (copy number of transgene per haploid genome) and than multiplied with 2 or 4 to express copy number of transgene per cell (copy number of transgene per diploid or tetraploid genome).

Validation of analytical method
The objective of validation of an analytical procedure is to demonstrate its suitability for the intended purpose (3). The validity of a method should be verified by laboratory studies and specific acceptance criteria which are set in advance should be achieved. Information of successful completion of such studies should be provided in the assay validation report. In this case study particular assays were designed to determine the accuracy, precision (intra-assay, intermediate), specificity, selectivity, linearity, the limit of detection, the limit of quantification, range of quantification and test ruggedness and robustness of the developed real-time qPCR method for determination of transgene copy number. On the basis of data collected during method qualification acceptance criteria for above listed assays were set.

Evaluated parameters

Accuracy
The degree of closeness of the determined value to the nominal or known true value under prescribed conditions (2). Two approaches of determining accuracy are available. The first is application of an analytical procedure to a reference material. The second is comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure.
In the case of copy number determination accuracy testing is only possible by using reference material (Table1). Reference material was prepared by combining certain amount of genomic and plasmid DNA to obtain defined ratios in copy number (1:25, 1:50, 1:100). Accuracy is reported as the difference between the measured and calculated values (recovery) in %.

Reference material		Copy number of transgene in reaction	Copy number of endogenous gene in reaction	Ratio of transgene copy number per one copy of endogenous gene
A	Calculated	133766	5095	26
	Measured	133006	5258	25
	Difference (%)	0.57	3.20	3.85
B	Calculated	206072	3925	53
	Measured	223122	4155	54
	Difference (%)	8.27	5.86	1.89
C	Calculated	651682	6206	105
	Measured	654464	6345	99
	Difference (%)	0.43	2.24	5.51
Acceptance criterion recovery (%) ≤ 30%				

Table 1: Test of accuracy on prepared reference material (combination of genomic and plasmid DNA in certain ratio). Differences between calculated and measured values are presented in %.

Precision (intra-assay, intermediate)
Precision is defined as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions (2).

Intra-assay precision or repeatability expresses the precision under the same operating conditions over a short interval of time (1).

Intra-assay precision was determined by comparing Ct values (Table 2), copy number per reaction among parallels for 5 dilutions of sample (Table 3), copy number per cell among 5 dilutions of sample (Table 4) and among 3 repeats of analysis in one run (Table 5). CV in % was calculated and used as measure of scatter.

Ct endogenous gene	Dilution 1 10x	Dilution 2 50x	Dilution 3 100x	Dilution 4 500x	Dilution 5 1000x
1.parallel	23.61	26.24	27.56	29.94	31.21
2.parallel	23.64	26.17	27.43	30.05	30.78
3.parallel	23.70	25.94	27.48	30.04	30.76
Average	23.65	26.12	27.49	30.01	30.92
SD	0.05	0.16	0.07	0.06	0.25
CV (%)	0.19	0.61	0.24	0.20	0.82

Ct transgene	Dilution 1 10x	Dilution 2 50x	Dilution 3 100x	Dilution 4 500x	Dilution 5 1000x
1.parallel	18.24	20.76	22.15	24.40	25.50
2.parallel	18.41	20.68	21.88	24.43	25.49
3.parallel	18.29	20.62	21.84	24.28	25.67
Average	18.31	20.69	21.96	24.37	25.55
SD	0.09	0.07	0.17	0.08	0.10
CV (%)	0.48	0.34	0.78	0.32	0.40
Acceptance criterion CV (%) ≤ 2%					

Table 2: Test of intra-assay precision. Ct values were compared among parallels for 5 dilutions of sample. CV in % is a measure of scatter.

Copy number of endogenous gene in reaction	Dilution 1 10x	Dilution 2 50x	Dilution 3 100x	Dilution 4 500x	Dilution 5 1000x
1.parallel	8720	1580	671	143	63
2.parallel	8545	1653	730	133	83
3.parallel	8226	1923	707	134	84
Average	8497	1718	703	137	77
SD	251	180	29.72	5.48	12
CV (%)	2.95	10.50	4.23	4.01	15.59

Copy number of transgene in reaction	Dilution 1 10x	Dilution 2 50x	Dilution 3 100x	Dilution 4 500x	Dilution 5 1000x
1.parallel	109606	20084	7912	1752	834.3043
2.parallel	97788	21198	9508	1716	838.2512
3.parallel	106143	22068	9771	1893	743.1098
Average	104513	21117	9064	1787	805.2218
SD	6075	995	1006	93	54
CV (%)	5.81	4.71	11.10	5.23	6.68
Acceptance criterion CV (%) ≤ 20%					

Table 3: Test of intra-assay precision. Copy number values were compared among parallels for 5 dilutions of sample. CV in % is a measure of scatter.

	Ratio of transgene copy number per cell (diploid)
Dilution 1	24.60
Dilution 2	24.57
Dilution 3	25.79
Dilution 4	26.17
Dilution 5	21.03
Average	24.43
SD	2.03
CV (%)	8.30
Acceptance criterion CV (%) ≤ 20%	

Table 4: Test of intra-assay precision. Copy number per cell was compared among 5 dilutions of sample. CV in % is a measure of scatter.

	Ratio of transgene copy number per cell (diploid)
Repeat 1	26.45
Repeat 2	25.22
Repeat 3	28.53
Average	26.73
SD	1.68
CV (%)	4.56
Acceptance criterion CV (%) ≤ 20%	

Table 5: Test of intra-assay precision. Copy number per cell was compared among 3 repeats of analysis in one run. CV in % is a measure of scatter.

Intermediate precision expresses the precision affected by random events.

Intermediate precision was determined by comparing 7 repeats of analysis in 7 runs (Table 6). CV in % was calculated and used as measure of scatter.

	Ratio of transgene copy number per cell (diploid)
Plate 1	24.43
Plate 2	23.22
Plate 3	30.42
Plate 4	23.50
Plate 5	26.45
Plate 6	25.04
Plate 7	32.56
Average	26.52
SD	3.61
CV (%)	13.62
Acceptance criterion CV (%) ≤ 20%	

Table 6: Test of intermediate precision. Copy number per cell was compared among 6 repeats of analysis in 6 runs. CV in % is a measure of scatter.

Specificity and selectivity

Specificity is the ability of an analytical method to measure one defined analyte.

Specificity of the method was proven by testing the ability of the method to amplify and detect only the specific target sequence by using specific oligonucleotides for transgene and for endogenous gene crosswise on sample DNA, recombinant plasmid DNA and genomic DNA from nontransfected CHO cell.

- Ct values (Table 7) were determined and compared with predicted values.
- Tm °C (melting temperature) value is amplicon specific and was determined by using SybrGreen chemistry (Table 8). Determined Tm values were compared with predicted values.
- The exact sequence of the PCR product was obtained by sequencing and was compared to the predicted sequence (Table 9).

Ct	Transgene	Endogenous gene
Sample Acceptance criterion	18.5 Ct < 25	24 Ct < 25
Plasmid DNA Acceptance criterion	15.5 Ct < 20	Undetermined Ct > 30
Genomic DNA from CHO Acceptance criterion	34 Ct > 30	24 Ct < 25

Table 7: Test of specificity. Specific oligonucleotides for transgene and for endogenous gene were used crosswise on sample, plasmid and genomic DNA. Agreement between measured and predicted Ct values is a measure of specificity.

Tm °C	Transgene	Endogenous gene
Sample Acceptance criterion	78.4 78-79	80.4 80-81
Plasmid DNA Acceptance criterion	78.4 78-79	/ /
Genomic DNA from CHO Acceptance criterion	/ /	80.4 80-81

Table 8: Test of specificity. Tm values are verified by using SybrGreen chemistry. Agreement between measured and predicted Tm values for specific amplicon is a measure of specificity.

Sequencing of qPCR product	Agreement
Amplicon of transgene on plasmid	100%
Amplicon of transgene in sample	100%
Amplicon of endogenous gene in sample	100%
Acceptance criterion 100% agreement with predicted sequence	

Table 9: Test of specificity. Exact sequences were gained by sequencing. Agreement between obtained and predicted sequences was confirmed.

Selectivity is the ability of a analytical method to measure and differentiate the analytes in the presence of components that are present or are expected to be present.

Selectivity of the method was proven by testing the ability of the method to amplify and detect the specific target sequence in a mixture of recombinant plasmid and genomic DNA from nontransfected CHO cells. The difference in Ct values between individual DNA and the DNA mixtures was determined.

Ct for transgene	
Plasmid + water	14.39
Plasmid + extracted genomic DNA from CHO	14.335
Difference	0.055

Ct for endogenous gene	
Extracted genomic DNA from CHO + water	18.6
Extracted genomic DNA from CHO + plasmid	18.53
Difference	0.07
Acceptance criterion Ct<1	

Table 10: Test of selectivity. The ability of the method to detect the specific target sequence in DNA mixtures was tested. The difference in Ct values between individual DNA and DNA mixture was determined.

Linearity
The linearity of an analytical procedure is its ability (within a given range) to obtain results which are directly proportional to the concentration (amount) of analyte in the sample (1).

Standard curve of recombinant plasmid
The plasmid with a concentration of 100ng/µl is serially diluted in 10-fold steps. A standard curve is prepared using dilutions from 1ng/µl to 10atto/µl. Lower limit of linearity is the Ct value where the SD of triplicates exceeds 0.3, and the upper limit of linearity is a Ct value lower than 10.

Standard curve of genomic DNA of nontransfected CHO cells
Genomic DNA with a concentration of 100ng/µl is serially diluted in four 10-fold steps to a final concentration of 10pg/µl and two dilutions 50x and 500x are prepared from intermediate dilutions. Lower limit of linearity is a Ct value where the SD of triplicates exceeds 0.3 and the upper limit of linearity is the Ct value where inhibition of the PCR reaction caused by the DNA load is not present.

Dilution curve of genomic DNA of sample (transfected CHO cells)
Dilutions are prepared in the same way as for standard curve of genomic DNA of nontransfected CHO cells. Prepared standard curves for recombinant plasmid, genomic DNA of nontransfected CHO cells and dilution curve of genomic DNA of sample have to fulfil in Table 11 listed acceptance criteria.

	Slope	Efficency of amplification	R^2	Number of log units	Copy number at lower limit of linearity	Copy number at upper limit of linearity
Plasmid transgene Acceptance criterion	-3.4124 -3.1-(-3.8)	96.34 110%-80%	0.9997 0.99-1	4 3-6	513 ≥10	5128601 ≤10^8
Genomic DNA of nontransfected CHO cells endogenous gene Acceptance criterion	-3.4717 -3.1-(-3.8)	94.12 110%-80%	0.9995 0.99-1	2 2-3	99 ≥10	9896 ≤10^4
Genomic DNA of sample transgene endogenous gene Acceptance criterion	-3.4255 -3.4457 -3.1-(-3.8)	95.85 95.08 110%-80%	0.9979 0.997 0.99-1	2 2 2-3		

Table 11: Test of linearity. Standard curves for recombinant plasmid, genomic DNA of nontransfected CHO cells and dilution curve of genomic DNA of sample have to fulfil listed criteria.

Limit of detection (LOD)
LOD is the lowest concentration of an analyte that the analytical procedure can reliably differentiate from background noise (2).

The lower limit of detection is the Ct value which is at least one cycle above the Ct value of NTCs (No Template Controls) in each parallel of triplicate.

Limit of quantification (LOQ)
Lower limit of quantification (LLOQ) is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy (2).

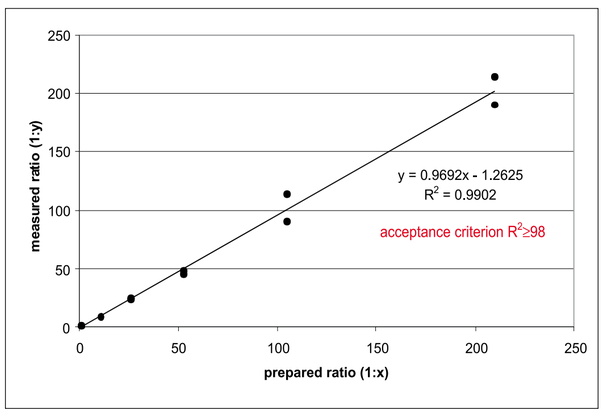
The lower limit of detection is the Ct value where the SD of triplicates does not exceeds 0.3, it is at least 3 cycles above the Ct value of NTCs (No Template Controls) and is not higher than 30.

Upper limit of quantification (UPOQ) is the highest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy (2).

Upper limit of quantification is the where the Ct value is lower than 10 and where inhibition of the PCR reaction caused by the DNA load is not present.

Range of quantification
A Range of quantification is The range of concentration, including ULOQ and LLOQ that can be reliably and reproducibly quantified with accuracy and precision through the use of a concentration-response relationship (2).

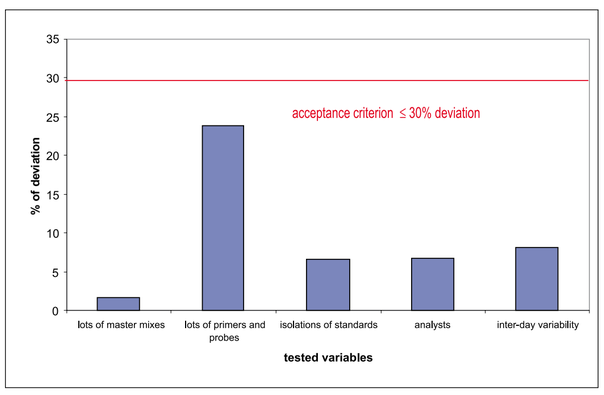
By using reference material (prepared by combining genomic DNA of nontransfected CHO cells and plasmid) it is demonstrated, that the method enables copy number determination from one copy to 200 copies of transgene per one copy of CHO cell genome, taking into account lower and upper LOQ. In this range we expected the copy number of transgene per haploid genome (i.e. two to 400 copies per diploid cell).The regression curve is constructed to determine the relationship between the calculated and measured ratio of reference materials. The criterion for linearity within the quantification range is the correlation factor (R^2≥0.98).



Graph 1: Determination of quantification range. Quantification range was demonstrated from 1 to 200 copies of transgene per one copy of CHO cell genome.

Ruggedness (reproducibility in ICH guideline(1))
The ruggedness of an analytical procedure is a measure of its capability to remain unaffected by normal, expected variations in method parameters during normal usage of the method. Variable method parameters are different analysts, different days of analysis, different equipment, different lots of reagents …)
The following variations were examined:

- influence of two different lots of master mixes, two different lots of primers and probes
- influence of two different extractions of plasmid and genomic DNA from nontransfected CHO cells used as standards
- influence of two different analysts, who performed the whole analytical procedure
- influence of inter-day repeatability, the analysis was performed on two different days

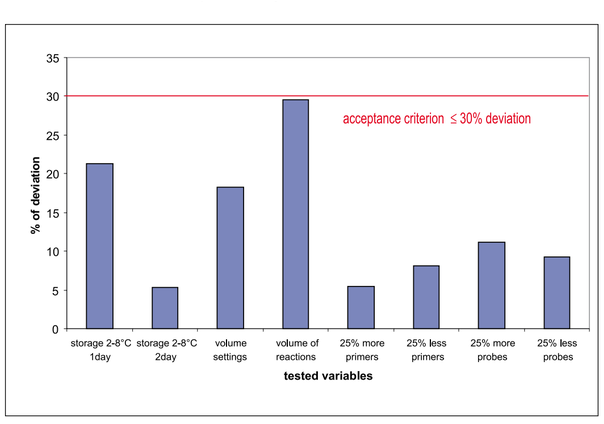


Graph 2: Ruggedness of the method. Influence of different lots of reagents: master mixes, primers and probes, different extractions of standards, different analysts and inter-day variability were tested. Deviations in copy number of transgene per cell were calculated between reference and tested variable and are expressed in %.

Robustness
The robustness of an analytical procedure is a measure of its capability to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage (1).

The following variations were examined:

- influence of storage of prepared qPCR reactions in a refrigerator 2-8°C before analysis for 1, 2 and 3 days
- influence of volume setting on qPCR apparatus, 20µl instead of 9µl
- influence of volume of qPCR reaction, 18µl instead of 9µl
- influence of different concentrations of primers and probes, 25% more and 25% less primers and probes in final reaction volume
- influence of master mixes produced by two different manufacturers



Graph 3: Robustness of the method. Influence of storage at 2-8°C, volume setting, volume of reaction, 25% more and 25% less primers and probes in reaction were tested. Deviations in copy number of transgene per cell were calculated between reference and tested variable and are expressed in %.

Conclusions

We confirmed that the method for transgene copy number determination by real-time qPCR can be validated appropriately with the proposed assays for the above listed parameters. Based on the presented work a validation protocol will be prepared, which subsequently will be used in our laboratory to verify the method for GMP use. In this way reliability of results obtained by the developed real-time qPCR method for transgene copy number determination is ensured.

Referencess

1. ICH-Validation of Analytical Procedures: text and methodology Q2(R1). *Federal Register* 1995, Vol.60, pages 11260 and 1996, Vol.62, No.96, pages 27463-27467.
2. CDER (Center for drug evaluation and research)-Guidance for Industry. Bioanalytical Method Validation. Bethesda MD: FDA; 2001.
3. CDER (Center for drug evaluation and research)-Draft Guidance for Industry. Analytical Procedures and Methods Validation. Bethesda MD: FDA; 2000.