Quantitation of residual DNA in final product biopharmaceuticals using the Threshold Total DNA assay system. Assay validation and determination of detection limit.

INTRODUCTION

The FDA recommends that all quality control tests used for biopharmaceutical product release follow current Good Manufacturing Practices (cGMP's) outlined in the Code of Federal Regulations Title 21. Validation of a quality control test must demonstrate that it provides acceptable performance with respect to accuracy, precision, linearity (characterization of the assay response curve, including sensitivity), detection limit, system suitability (only required for chromatographic assays) and specificity[1]. Tests used to quantitate residual DNA in final product biopharmaceuticals (e.g. Threshold® Total DNA assay, hybridization, etc.) fall into this category and should be validated acording to these criteria.

Since linearity and specificity have been previously characterized for the Threshold Total DNA assay[2], the other relevant performance criteria that remain to be evaluated prior to routine DNA quantitation using the Threshold System are accuracy, precision and detection limit. Accuracy can be demonstrated through evaluation of the recovery of DNA added to the sample (spike recovery). Precision can be evaluated through within-day, day-to-day, operator-to-operator and laboratory-to-laboratory performance studies. The detection limit will be a function of the sample pretreatment method employed and the confidence level (95%, 99.9%, etc.) required.

This application note suggests one technique for validating quantitation accuracy. In addition, techniques are suggested for quantitation of residual DNA and determination of the detection limit in actual biopharmaceutical samples and buffers. In this study, it is assumed that the most efficient pretreatment protocol has already been established[3] and that the proper DNA standard has been chosen, prepared and qualified[4].

MATERIALS & METHODS

Final product (formulated or bulk, pre-formulated) biopharmaceuticals (human growth hormone [rHGH]; IgG and protein X) were supplied by Threshold users. Two percent (w/v) solutions of SDS (Bio-Rad Electrophoresis grade) were prepared in Zero Calibrator (50 mM Phosphate buffered saline at pH 7.0, provided in the Threshold Total DNA assay kit). Proteinase K [Boehringer Mannheim Biochemicals (Indianapolis, IN)] solutions at 2 mg/ml were also prepared in Zero Calibrator.

In this study, samples containing a known amount of added DNA (a spike) were prepared by: 1) serial dilution of a stock DNA solution into an appropriate diluent (e.g. control buffer or 1 mg/ml protein solution for the IgG and rhGH studies); or 2) individually spiking samples (e.g. control buffer or 1.6 mg/ml protein solution for protein X) with known amounts of DNA. All samples were prepared in triplicate and protease digested overnight in individual sterile screw-capped tubes. Aseptic technique was observed in all sample handling steps prior to the DNA heat-denaturation step.

A list of suggested and required materials (e.g. sterile pipet tips and tubes) for use in the pretreatment and assay of samples on the Threshold System is supplied in the *Threshold System Operator's Manual*. Threshold instruments and reagent kits were supplied by Molecular Devices Corporation, Sunnyvale, CA. The protocols outlined in the *Threshold System Operator's Manual* for protein digestion, preparation of normal standard curves and Threshold System and software operation were followed in all experiments performed.

RESULTS & DISCUSSION

The Threshold bioanalytical system software, THS, calculates DNA concentration in units of mass per 0.5 ml sample. The resulting quantitated values will include DNA that may have been introduced during the pretreatment steps. This exogenous DNA should be subtracted before reporting the level of DNA per mg of biopharmaceutical product. Thus, when proper standards and controls are included, quantitation of residual DNA in the sample can be calculated manually. Quantitation accuracy can be assessed by evaluating the percent recovery of DNA added to an unknown sample relative to buffer controls treated in a manner identical to that of the unknown sample.

Validation of pretreatment method

In validating quantitation accuracy, it is important to show that the shape and slope of the standard curve is unaffected (within the experimental error of the test method) by the pretreatment method and/or the sample constituents. One method to determine the effect of the pretreatment method on DNA recovery, is to compare a standard curve prepared from heat denatured (single-stranded) DNA ("normal standard curve", as suggested in the *Threshold System Operator's Manual*) to one prepared from double-stranded DNA in which the samples have been treated by the proposed sample pretreatment method. Figure 1 shows an example of such a comparison. The pretreatment method in this case consisted of an overnight treatment with SDS and Proteinase K at 55°C. Acceptable spike recovery is usually defined as $100 \pm 20\%$ (depending upon laboratory preference) when compared to an appropriate control sample. Linear regression analysis of the standard curves for the comparison shown in Figure 1 is sufficient because only the low, linear end of the standard curves are being examined. Regression analysis gives the following equations for the buffer and the normal standard curve:

y = 0.84 x + 6.3   R2 = 0.998   buffer

y = 0.82 x + 1.2   R2 = 0.999   normal standard curve

If the spike recovery guidelines listed above are followed, each treated sample point should be within 20-30% of its corresponding control. Thus, the two lines should be roughly parallel. Comparison of the slopes of the two lines reveals that they differ by only 2.5%. Moreover, both regression coefficients are greater than 0.998, thus this linear analysis is valid and the results show that the shape of the standard curve is unaffected by the pretreatment method and associated reagents used.
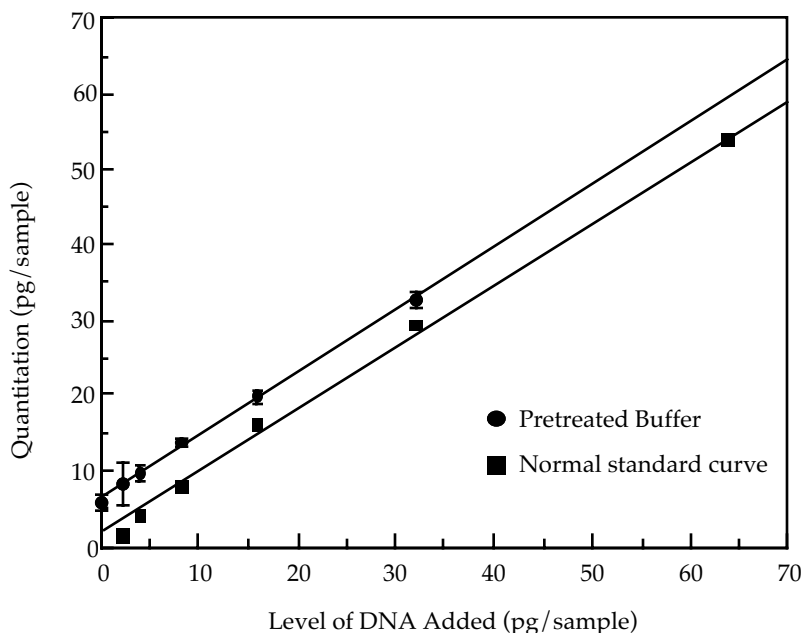

Figure 1: Comparison of pretreated buffer and normal standard curves. Quantitation of pretreated buffer standard curve points was based on the normal standard curve. Pretreated samples were prepared as bulk solutions, separated into triplicates for overnight digestion and then assayed the next morning. Pretreatment consisted of overnight digestion at 55°C in the presence of 0.1% SDS and 100 μg/ml Proteinase K.

Determination of exogenous DNA that may be introduced by the pretreatment method and its associated reagents can be calculated by taking the difference in y-intercept between the pretreated buffer and the normal standard curve. In this example, the difference is 5.1 pg. Thus, 5.1 pg of DNA was introduced during sample pretreatment. This amount should be taken as the baseline in manual calculations to determine residual DNA in biopharmaceutical samples that have been treated in a similar manner and in the same experiment.

Validation and quantitation of residual DNA in samples

Using a similar analysis, a pretreated biopharmaceutical sample can be compared to the pretreated buffer controls. Figures 2 and 3 present such a comparison for final product IgG and rhGH samples.   Linear regression analysis shows that the regression coefficients are greater than 0.97 for all lines. In both cases, the difference in quantitation between points for the buffer controls and the protein samples is negligible (within the acceptable statistical error). Thus, the regression lines for the pretreated sample and the pretreated buffer are roughly parallel.

Also,, the difference in y-intercept for both proteins is ≤ 1 pg. Because of these facts and for simplicity, in these graphs, one line is used to represent the data for all pretreated samples.
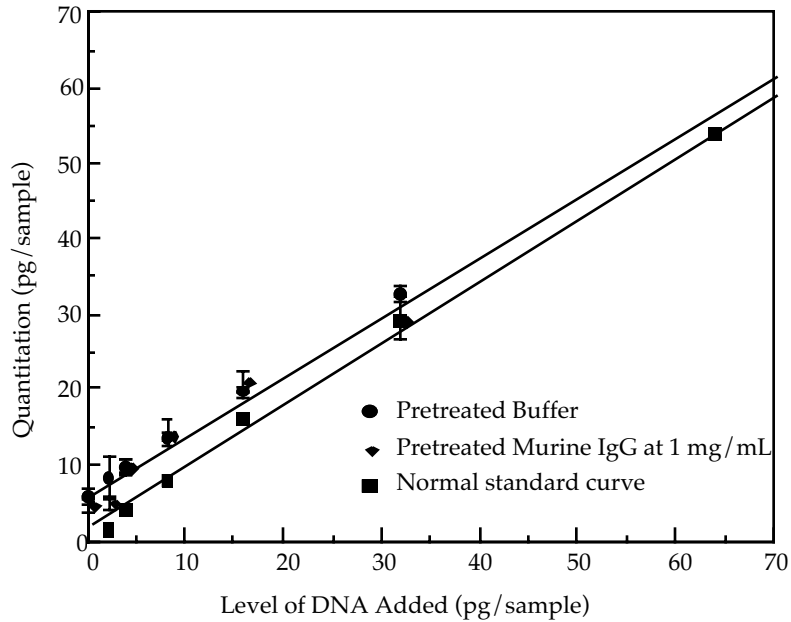

Figure 2: Comparison of pretreated buffer and pretreated final product IgG sample to a normal standard curve. Quantitation of pretreated buffer sample was based on the normal standard curve. Pretreated samples were prepared as bulk solutions, separated into triplicates for overnight digestion and then assayed the next morning. Pretreatment consisted of overnight digestion at 55°C in the presence of 0.1% SDS and 100 μg/ml Proteinase K.
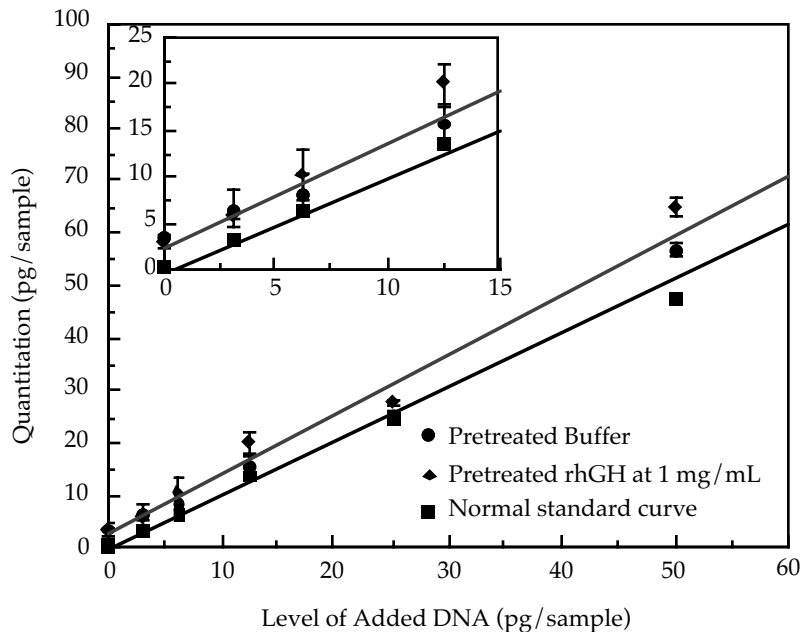

Figure 3: Comparison of pretreated buffer and pretreated final product rhGH to a normal standard curve. Quantitation of pretreated buffer sample was based on the normal standard curve. Pretreated samples were prepared as bulk solutions, separated into triplicates for overnight digestion and then assayed the next morning. Pretreatment consisted of overnight digestion at 55°C in the presence of 0.1% SDS and 100 μg/ml Proteinase K.

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In both cases, the level of DNA can be reported as being less than the detection limit of the method. The sample constituents, in these two cases, do not affect the recovery of added DNA and as a result accurate quantitation is validated.

Once the assay quantitation accuracy is validated based on acceptable recovery at multiple spike levels, routine quantitation can be performed by testing replicates (e.g. duplicates or triplicates) of the sample and its appropriate buffer control and calculating the difference. However, it is recommended, that even in routine testing, one or two appropriate spike levels should be included as internal controls. When high sensitivity is required or detection limit is a problem, the testing of replicates at multiple spike levels in the low end of the standard curve is recommended. This will allow determination of the y-intercepts and resulting residual DNA level (difference in y-intercepts between the sample and the buffer control lines) with a higher degree of confidence.

Detection Limit

The detection limit for DNA in Zero Calibrator using the Threshold System is 2 pg $\pm$ 1 pg[2]. When biopharmaceutical samples are tested, pretreatment methods must usually be employed to adequately remove sample interferences[3]. The pretreatment method(s) and sample constituents can affect the detection limit of the system. The detection limit for a sample and its required pretreatment may be determined by testing replicates at multiple spike levels in the low end of the standard curve (≤ 10 pg). Table 1 presents one example of how to determine the limit of detection for assaying DNA in 1.6 mg of protein X following an overnight protease digestion.

Sample	Signal (μV/sec)	Standard deviation	CV (%)
1.6 mg protein X + 0 pg	35.5	2.3	6.5
1.6 mg protein X + 2 pg	50.5	4.3	8.5
1.6 mg protein X + 4 pg	61.9	5.9	9.5
1.6 mg protein X + 5 pg	67.8	7.3	10.8
1.6 mg protein X + 10 pg	95.2	3.5	3.7
1.6 mg protein X + 20 pg	168.0	9.7	5.8

Table 1: Example of detection limit determination

The following definitions of detection limit confidence level are commonly used in immunoassay development:

99.9% confidence level: positive - 2 std. dev. > negative + 2 std. dev.

95% confidence level: positive - 1 std. dev. > negative + 1 std. dev.

Using these definitions, the following calculation can be made for this sample:

2 pg - 2 std. dev. = 50.5 - 8.6 = 41.9 > 40.1 = 35.5 + 4.6 = 0 pg + 2 std. dev.

Based on this result, 1.6 mg of protein X can be assayed using an overnight digestion at a detection limit of 2 pg with 99.9% confidence. Thus, in reporting quantitation of residual DNA in this sample, if no difference is seen between the

sample and the appropriate control buffer, the level of residual DNA can be reported as less than 2 pg DNA per 1.6 mg protein or less than 1.25 pg/mg protein. In general, pretreatment requiring an overnight digestion will provide a detection limit in the range of 2 - 5 pg per 0.5 ml sample, while, because of the extra sample handling required, overnight digestion followed by DNA extraction will usually provide detection limits in the range of 5 - 10 pg per 0.5 ml sample.

In the above analysis, "raw" signals, in triplicate, spread over different sticks were used without adjustment for stick-to-stick variations ("stick gain"). This gives a more conservative estimate of the detection limit. Usually, it is best to first adjust the signals by dividing by the stick gain before determining the detection limit. This gain adjustment will be important when the range of gain values for the sticks is large.

SUMMARY

Residual DNA can be manually quantitated using linear regression analysis of data obtained by spiking replicate samples and controls at multiple levels and taking the difference of the y-intercepts of the two lines (sample and control). This analysis is valid only in the low, linear portion of the standard curve. The values used in the analysis can be based on quantitation using a normal standard curve. Thus, pretreatment of the standard curve and the associated on-stick calibrators is not required and is not generally recommended. Levels measured above the background associated with the pretreatment method itself should be confirmed with DNase I treatment and/or a dilution series of the sample.

This application note demonstrates one technique for validating the quantitation accuracy of the test method used to determine residual DNA levels in final product biopharmaceutical samples. Suggestions have been presented for methods to determine both the quantity of residual DNA and the detection limit for a particular sample with its associated pretreatment method. These suggestions should not be considered as strict guidelines which all users must follow, but are to be viewed solely as guidelines for efficient application of the Threshold System

REFERENCES

1 Williams, D. F. An overview of test method validation. *BioPharm Mfg* **1:**34-36, 51(1987).

2 Kung V. T., Panfili, P.R., Sheldon, E. L., King, R. S., Nagainis, P. A., Gomez, B., Ross, D. A., Briggs, J., and Zuk, R. F. Picogram quantitation of total DNA using DNA-binding proteins in a silicon sensor-based system. *Anal. Biochem.* **187**:220-227 (1990).

3 Threshold Total DNA application note "Adapting high protein, low DNA samples for Threshold Total DNA analysis", Molecular Devices Corporation (1989).

4 Threshold Total DNA application note "Preparation and qualification of DNA standards for use in the Threshold Total DNA assay", Molecular Devices Corporation (1990).