

DNase I qualification and sample treatment

INTRODUCTION

The Threshold Total DNA Assay quantitates contaminant DNA in biopharmaceutical drugs. DNase I can be used in conjunction with the Total DNA Assay for two purposes:

To validate that the assay signal and subsequent quantitation generated by the Total DNA Assay specifically measures DNA and not a non-DNA component of the sample, or

To confirm that observed inhibition of the Total DNA Assay is caused by the presence of small fragment DNA, rather than protein or buffer interference.

This application note describes procedures to address both of these applications. A method is also provided to qualify a new lot of DNase I.

DNase I is a 37,000 Dalton endonuclease derived from bovine pancreas that degrades double-stranded DNA to produce 3'-hydroxyl oligonucleotides. The enzyme requires divalent cations for activity and the specificity of the reaction depends upon which divalent cation is present. Mg^{2+} was selected because, in its presence, the DNase I nicks each strand of double-stranded DNA independently, generating random cleavage sites¹.

MATERIALS

- 1 ***Threshold[®] System*** from Molecular Devices Corporation (catalog #0200-0500), 1311 Orleans Drive, Sunnyvale, CA 94089, tel: 408-747-1700 or 800-635-5577.
- 2 ***Total DNA Assay Kit*** from Molecular Devices Corporation (catalog #R9009).
- 3 ***Total DNA Zero Calibrator Kit*** from Molecular Devices Corporation (catalog #R8004).
- 4 ***DNase I, RNase free, from bovine pancreas*** from Boehringer Mannheim Corp. (catalog # 776-785).
- 5 ***Bovine serum album, special quality for molecular biology***, from Boehringer Mannheim Corp. (catalog # 711-454).

- 6 *Glycerol, ultrapure molecular biology grade*, from Boehringer Mannheim Corp. (catalog # 100-647).
- 7 *EDTA, disodium salt*, from Sigma Chemical Co. (catalog # E5134).
- 8 *MgCl₂*, from Sigma Chemical Co. (catalog # M2670).
- 9 *Tris (hydroxymethyl) aminomethane hydrochloride, ACS grade*, from Aldrich Chemical Company, Inc. (catalog # 85, 764-5).
- 10 *Dithioerythritol (DTE.)*, from Aldrich Chemical Company, Inc. (catalog #16,176-4).

METHODS

DNase I is added to a sample for testing prior to heat denaturation. Therefore it is necessary to practice good aseptic technique to minimize contamination with non-sample DNA. Sterile, individually wrapped pipet tips, sterile tubes, and gloves should be used throughout this procedure.

Because DNase I may exhibit lot-to-lot variation, it is important to qualify the activity of each lot prior to its use with samples. The optimal amount of DNase I will reduce the quantitation of a 50 pg spike of double stranded DNA by at least 90%. Excess DNase I in the presence of free Mg²⁺ ions may slow or stop sample filtration in the Total DNA Assay. For this reason, EDTA should be added to each sample after the digestion to chelate all free Mg²⁺ ions.

DNase I qualification

Three levels of DNase I are recommend for the initial testing: 25, 50 and 100 activity units (U).

- Step 1** Prepare a stock solution of DNase I at 2500 U/mL in the following buffer: 20 mM Tris-HCl, 1 mM dithioerythritol, 50 mM NaCl, 0.1 mg/mL BSA, 50% glycerol. Aliquot and store at -20°C.
- Step 2** Prepare a 100 mM solution of MgCl₂ in Zero Calibrator. Pass through a 0.2 µm filter then store at room temperature in a sterile Corning centrifuge tube.
- Step 3** Prepare a 160 mM solution of EDTA in Zero Calibrator or sterile water-for-injection. Pass through a 0.2 µm filter then store at room temperature in a sterile Corning centrifuge tube.
- Step 4** Prepare a 1 ng/mL DNA solution for the spike addition by diluting 200 µL of High Calibrator into 800 µL of Zero Calibrator.
- Step 5** Label duplicate sample tubes as follows and pipet 0.5 mL of Zero Calibrator (ZC) into each tube:

No DNase	25 U DNase	50 U DNase	100 U DNase
(2) ZC +spike	(2) ZC +spike	(2) ZC +spike	(2) ZC +spike
(2) ZC - spike	(2) ZC - spike	(2) ZC - spike	(2) ZC - spike

There should be a total of 16 tubes for the DNase I qualification.

- Step 6** Add 10 μL , 20 μL , and 40 μL of the DNase stock solution to each of the tubes for 25 U, 50 U, and 100 U of DNase, respectively.
- Step 7** Add 50 μL of the double-stranded 1 ng/mL DNA solution to each of the tubes designated for spike addition (+spike).
- Step 8** Add 25 μL of 100 mM MgCl_2 solution to each of the tubes containing DNase I. Cap tightly and vortex to mix.
- Step 9** Incubate 3 hours at 37°C.
- Step 10** Heat samples at 105°C for 15 minutes, and cool on ice for 5 minutes.
- Step 11** Add 50 μL 160 mM EDTA solution to each of the tubes containing DNase I.
- Step 12** Analyze the denatured samples with the Total DNA Assay. The lowest level of DNase I that reduces the DNA spike by at least 90% and still allows for proper filtration is the optimum concentration. However, some proteins may inhibit DNase I activity so that for these samples, a higher level of DNase I may be required.

Note: Additions to the sample tubes outlined in steps #7, 8, and 11 above can be made using an Eppendorf Repeater Pipetter with a sterile, individually wrapped 1.25 mL CombiTip[®] and a sterile pipet tip.

Sample pretreatment

Samples that may contain high concentrations of DNA should first be evaluated by the dilution test described in the Total DNA section of the *Threshold System Operator's Manual*. This procedure will determine if the sample contains DNA levels that exceed the dynamic range of the Total DNA Assay standard curve (3-200 pg/test) which can cause reduced spike recovery.

DNase I digestion must be done prior to a protease digestion. SDS inhibits DNase I and Proteinase K or Pronase will digest and inactivate the DNase I. If the sample must be digested be sure to also digest all the ZC controls.

The total amount of MgCl_2 to be added to the sample depends upon the amount of EDTA or Mg^{2+} present in the sample buffer (if any) and should be altered to achieve a final sample concentration of 5 mM Mg^{2+} for maximal activity of the DNase. To assure that an adequate amount of EDTA is added to each sample after digestion, determine if Mg^{2+} or other cations are present in the sample buffer. If so, increase the amount of EDTA to chelate all the cations.

Validating the specificity of the Total DNA assay

Step 1 Label the sample tubes, in duplicate, and aliquot 0.5 mL of each sample into the appropriate tube:

Controls	Samples
(2) ZC	(2) Sample
(2) ZC + spike	(2) Sample + spike
(2) ZC + spike + DNase I	(2) Sample + spike + DNase I
	(2) Sample + DNase I
(2) ZC + DNase I + post spike	(2) Sample + DNase I + post spike

The spiked Zero Calibrator (ZC) controls, with and without DNase I, are included to validate the activity of the enzyme. The spiked and non-spiked samples, with and without DNase I, are used to show that the signal measured in the sample is actually DNA. The samples tested with a post spike of denatured DNA are used to determine that the DNase only decreases signal by digesting DNA, and does not affect the assay by another mechanism.

Step 2 Prepare a 1 ng/mL DNA solution for the spike addition by diluting 200 μ L of High Calibrator into 800 μ L of Zero Calibrator.

Step 3 Add the selected level of DNase I stock solution to each of the tubes designated for DNase I addition.

Step 4 Add 25 μ L of 100 mM $MgCl_2$ solution to each of the tubes containing DNase I.

Step 5 Add 50 μ L of the double-stranded 1 ng/mL DNA stock to each of the tubes designated for spike addition (+ spike).

Step 6 Incubate a minimum of 3 hours at 37°C (some protein samples may require an overnight incubation for the DNase digestion to reach completion).

Step 7 Heat all samples at 105° C for 15 minutes, then cool on ice for 5 minutes.

Step 8 At the same time, heat denature the remaining 1 ng/mL DNA solution which will be used for the post spike addition and 500 μ L of High Calibrator which will be used to make a DNA standard curve. Chill on ice for 5 minutes.

Step 9 Add 50 μ L of 160 mM EDTA solution to each of the tubes containing DNase I.

Step 10 Add 50 μ L of denatured 1 ng/mL DNA solution to each of the tubes designated for post spike (+ post spike).

Step 11 Analyze the denatured samples using the Threshold Total DNA Assay. If all suggested controls and sample conditions are run, the assay will use four sticks (three sample sticks and one standard curve).

Note: Additions to the sample tubes outlined in steps #3, 4, 5, 9, and 10 above can be made using an Eppendorf Repeater Pipetter with a sterile, individually wrapped 1.25 mL CombiTip and a sterile pipet tip.

Confirming the presence of DNA fragments

Step 1 Label the sample tubes, in duplicate, and aliquot 0.5 mL of each sample into the appropriate tube:

Controls	Samples
	(2) Sample
(2) ZC + spike	(2) Sample + spike
(2) ZC + spike + DNase I	(2) Sample + spike + DNase I
(2) ZC + DNase I	(2) Sample + DNase I
2) ZC + DNase I + post spike	(2) Sample + DNase I + post spike

The spiked Zero Calibrator (ZC) controls, with and without DNase I, are included to validate the activity of the enzyme. The ZC denatured post spike DNA control is used as a comparison with the post spike sample. The sample tested with a post spike of denatured DNA is used to determine whether inhibition of the Total DNA Assay is due to DNA or to other inhibitory factors, such as protein or buffer constituents.

Step 2 Follow steps 2 through 11 listed in the previous section.

DATA ANALYSIS

Validating the specificity of the Total DNA Assay

Step 1 Calculate the net spike for the following controls and samples:

	Spiked	Non-Spiked	Net
ZC			
Sample			
ZC + DNase I		—	
Sample + DNase I			
ZC + DNase I + post spike		—	
Sample + DNase I + post spike		—	

Step 2 Compare the net spike quantitations (Net above) of the DNase digested ZC controls with the net spike quantitations of the untreated ZC controls.

If quantitation of net ZC + DNase is $\leq 10\%$ of net ZC, the DNase is active and performing satisfactorily.

Step 3 Compare the net spike quantitations of the DNase digested samples with the net spike quantitations of the untreated samples.

If quantitation of net sample + DNase is $\leq 10\%$ of net sample, the Total DNA Assay is measuring only DNA.

Step 4 Compare the spike quantitations of the samples which were DNase digested and post spiked with denatured DNA to the spike quantitations of the ZC which were DNase digested and post spiked.

If the net sample + DNase + post spike is 80-120% of the quantitation of the net ZC + DNase + post spike, none of the reagents added to the sample to perform the experiment do not interfere in the Total DNA Assay.

Confirming the presence of DNA fragments

Step 1 Calculate the net spike for the following controls and samples:

	Spiked	Non-Spiked	Net
ZC		—	
Sample			
ZC + DNase I			
Sample + DNase I			
ZC + DNase I + post spike		—	
Sample + DNase I + post spike		—	

Step 2 Compare the net spike quantitation (Net above) of the DNase digested ZC control with the spike quantitation of the untreated ZC control.

If quantitation of net ZC + DNase $\leq 10\%$ of spiked ZC, the DNase is active and performing satisfactorily.

Step 3 Compare the net spike quantitation of the non-DNase digested sample with the spike quantitation of the non-DNase digested ZC control.

If the sample has $< 80\%$ spike recovery of the ZC control, DNA detection is being inhibited. Proceed to step 4.

Step 4 Compare the net spike quantitation of the DNase digested, post-spiked sample (with the DNase digested, non-spiked sample quantitation subtracted out) with the net spike quantitation of the DNase-digested, post-spiked ZC control (with the DNase treated, non-spiked ZC quantitation subtracted out).

If the sample + DNase + post spike is 80-120% of the ZC + DNase + post spike, then DNA fragments or excess DNA is inhibiting the Total DNA Assay.

If the net sample + DNase + post spike is $< 80\%$ of the quantitation of the net ZC + DNase + post spike, the inhibited spike recovery may be due to buffer or protein inhibition rather than DNA fragments or high levels of DNA.

SUMMARY:

DNase I is very specific for DNA. Elimination of the signal observed in the Total DNA Assay by DNase I digestion will confirm the specificity of the Total DNA Assay. If the DNase I digestion fails to reduce the signal produced in the Total DNA Assay by 90% or more, pretreatment methods outlined in the *Threshold System Operator's Manual* should be investigated. Please note that when analyzing final product samples with less than 10 pg of DNA, it may be difficult to distinguish a 90% reduction in signal over background, especially in samples that have undergone a pretreatment procedure. The use of the DNase I procedure can also determine if observed inhibition of the Threshold Total DNA Assay is due to DNA, either an excessively high concentration of DNA or the presence of DNA fragments in the sample.

REFERENCES:

- 1 Maniatis, T., Fritsch, E.F., Sambrook, J. *Molecular Cloning, A Laboratory Manual*, 2nd edition; Cold Spring Harbor Laboratory Press (1989).
- 2 *Threshold System Operator's Manual*, Molecular Devices Corporation (1993).

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