

Process Development

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Detecting and quantifying type I endonuclease activity is important in several areas of biopharmaceutical product development. Measuring the activity of a product such as the recombinant human DNase Pulmozyme, used to treat cystic fibrosis, is one such application (1). Another important application is quantifying trace residual endonuclease levels in biopharmaceuticals that use a type I endonuclease to remove residual host-cell DNA during processing. One representative of type I endonucleases, benzon nuclease, is a broad-specificity extracellular protein isolated from *Serratia marcescens* that degrades both single- and double-stranded DNA and RNA with no apparent sequence specificity (2). Residual testing for endonucleases added to processes for removal of excess DNA is now critical in light of several recent publications suggesting that permeabilized mammalian

has superior sensitivity to the hyperchromicity assay, but the preparation of the substrate is time consuming, and the prepared substrate has a brief storage life. It does have a higher sample throughput, but in our tests it lacked the desired sensitivity to be used as a trace residual assay, and it had problems overcoming sample matrix interference. A commercially available ELISA kit uses an antibody specific for benzon nuclease in a capture-type format with a stated lower limit of quantitation of 0.5 ng/mL, equivalent to about 0.5 U/mL of benzon nuclease. Unfortunately, the kit is prohibitively expensive and offers no significant increase in sensitivity over the hyperchromicity method.

This article describes a homogeneous format (with no wash or separation steps), microplate-based quench fluorescence assay that uses molecular beacon technology to quantify type I endonuclease activity in a number of sample matrices. We then compare the sensitivity of this method with those of existing assays.

Molecular beacons are described in the literature as sensitive tools for detecting specific DNA target sequences (9). A molecular beacon is an oligonucleotide with a specific target probe sequence flanked on both its 3' and 5' sides by a complementary stem sequence terminating with a fluorophore at the 5'-end and a quench molecule at the 3'-end. When not hybridized to its target sequence, the molecule generates minimal fluorescence because its stem region maintains the fluorophore and quench in close proximity. However, when hybridized to its complementary target sequence, the molecular beacon generates a fluorescent signal proportional to the amount of target sequence present because it separates the quench from the fluorophore.

We hypothesized that a nonspecific nuclease would unquench the fluorescence of the beacon, providing the foundation for the beacon endonuclease (BEN) assay. The original substrate we investigated was a 34-mer with a 7-nucleotide stem region. Figure 1 provides a conceptual view of the BEN assay. Two additional experimental substrates that varied the oligonucleotide length (4 and 8 nucleotides) or eliminated the stem region (no stem or a two-nucleotide stem) were also evaluated in the BEN assay system. The advantage of those smaller oligonucleotides was that their smaller size

Using Molecular Beacons to Quantify Low Levels of Type I Endonuclease Activity

The ability to quantify residual type I endonuclease has been hampered by the lack of methods that possess the required performance, sensitivity, sample throughput, and economy. A novel use of molecular beacons may be the answer to this problem.

cells treated with benzon nuclease showed signs of chromosomal aberrations (3–5). This finding underscores the need for residual endonuclease assays with high sensitivity.

A number of assays are described in the literature for quantifying type I endonuclease activity. The standard hyperchromicity assay described by Kunitz for quantitating DNase I activity is a widely accepted assay but requires extensive manipulations, including an acid precipitation step before reading (6–7). The measurement step for a hyperchromicity assay is an A_{260} determination, which requires that samples and standards be read individually in a quartz cuvette. That requires extensive hands-on time during analysis and cannot easily be adapted to a high sample throughput format. Additionally, the method is relatively insensitive, and in our tests its lower limit of quantitation (LLOQ) was 0.7 U/mL of benzon nuclease.

Several alternatives to hyperchromicity assays have become available in the past few years. A higher throughput colorimetric-based assay described by Sinicropi exploits a color shift in methyl-green labeled DNA on enzymatic degradation (8). This assay

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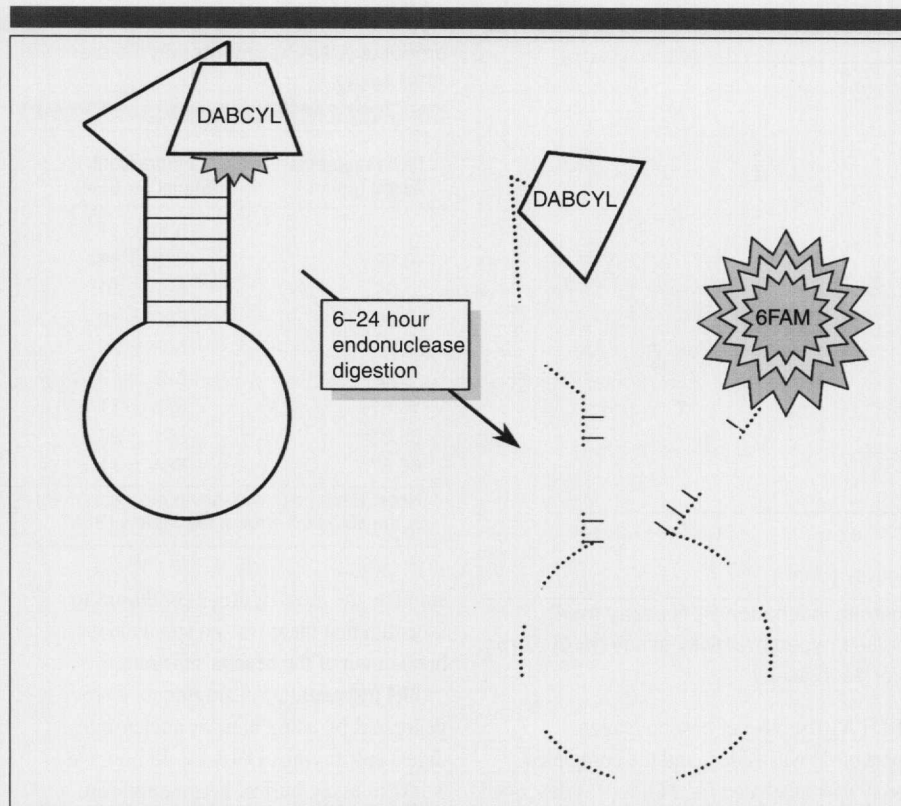


Figure 1. Cartoon depicting the rationale for the BEN assay (6FAM = 6-carboxyfluorescein).

allowed them to be synthesized less expensively because no extensive purification was required. This assay can quantify low levels of type I endonuclease activity in a number of sample matrices using commercially available reagents. To our knowledge, it is the most sensitive quantitative endonuclease activity assay reported to date.

Materials and Methods

Molecular beacons of three different lengths and sequences (Figure 2) were obtained commercially from the Midland Certified Reagent Company (Midland, TX). Benzonase was obtained from Nycomed Pharma (Uppsala, Sweden), and the Pulmozyme was purchased by prescription from Genentech (South San Francisco, CA). OmniCleave was obtained from Epicentre Technologies (Madison, WI). Stock Tris-HCl (1M, pH 7.5) solution was purchased from BioFluids, Inc. (Rockville, MD), and Dulbecco's phosphate buffered saline solution was purchased from Quality Biological (Gaithersburg, MD). HPLC-grade reagent water was used for all solution preparations and was obtained from VWR Scientific (West Chester, PA). Black 96-well OptiPlates came from Packard

Instrument Company (Meriden, CT), and 96-tube dilution boxes were obtained from United Laboratory Plastics (St. Louis). The Cytofluor 4000 reader was purchased from PE Biosystems (Foster City, CA). The humanized recombinant monoclonal antibody Synagis came from MedImmune (Gaithersburg, MD). Unless specified, all other reagents were procured from the Sigma Chemical Company (St. Louis).

BEN protocol. Endonuclease stock was diluted in assay buffer (20 mM Tris, 2.5 mM MgCl₂, 0.1% w/v bovine serum albumin (BSA), pH 7.5) in a sterile 96-tube dilution box in 150 μ L volumes to construct a standard curve. Samples, spiked samples,

and controls were likewise diluted in assay buffer in the dilution box. The stock molecular beacon (beacon 1) was prepared in phosphate buffered saline (PBS) to a concentration of 14.5 μ M and was further diluted to 1:100 in assay buffer to prepare the working solution. Then, each tube in the dilution box received 150 μ L of that working solution using a 12-channel pipette. The box was sealed with Parafilm (Structure Probe, West Chester, PA), vortexed, and placed in an incubator in the dark at 37 $^{\circ}$ C, 100% humidity for the specified incubation period (6 or 24 hours). Before analysis, the box was taken from the incubator, vortexed, unsealed, and 200 μ L volumes were transferred to a black OptiPlate.

The plate was read in a Cytofluor 4000 plate reader with a 99-second shake cycle, then read with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The resulting data were exported to Excel (Microsoft, Redmond, WA) and analyzed with SOFTmax (Molecular Devices, Sunnyvale, CA) to generate a standard curve from which sample concentrations were obtained. The acceptance criteria for the BEN assay were a correlation coefficient ≥ 0.990 of the 4-parameter-fit standard curve, spike recovery for each sample between 75–125% of theoretical, and a positive control value (0.04 U/mL endonuclease spiked into assay buffer) within 75–125% of the expected value. The assay LLOQ was calculated by first taking the mean fluorescent signal from six wells containing assay diluent, representing the background. Then 10 standard deviations were added to that mean signal to produce the LLOQ signal. That signal was regressed on the endonuclease standard curve to give a concentration that was set as the LLOQ of the assay.

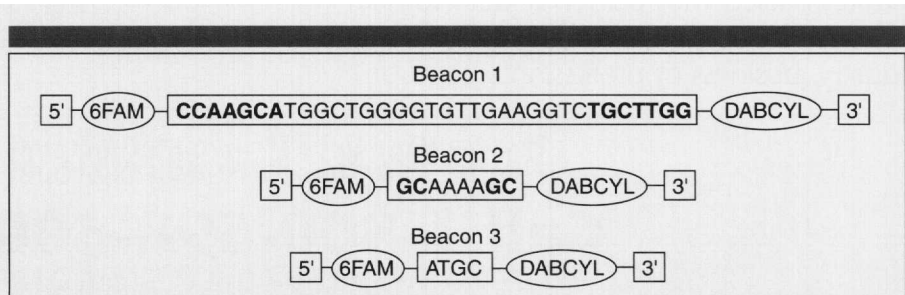


Figure 2. Sequence comparison of the three molecular beacons used in the BEN substrate studies. The bold nucleotides represent the double-stranded stem region of the beacon. (6FAM = 6-carboxyfluorescein)

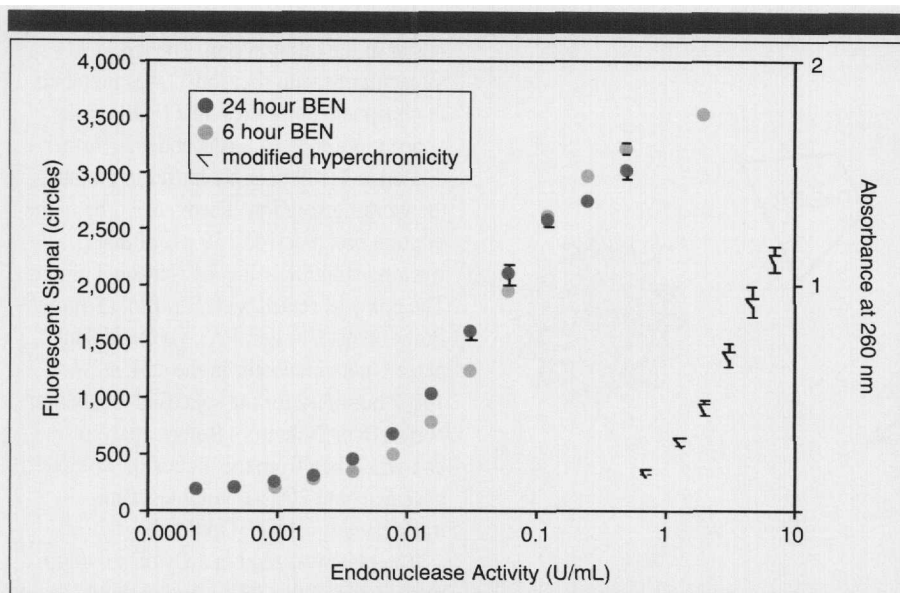


Figure 3. Comparison of the standard 24-hour substrate incubation BEN assay (n=4), 6-hour incubation BEN assay (n=3), and the modified hyperchromicity assay (n=6). Error bars represent the standard error of the means for each assay.

EDTA effects. Endonuclease standard curves were prepared in assay buffer containing 0, 1, or 5 mM ethylenediaminetetraacetic acid

(EDTA), the 34-mer beacon reagent (beacon 1) was added, and the completed assay was incubated for 24 hours as described above. The 24-hour incubation was chosen to

Table 1. Effect of benzon nuclease digestion products (DNA fragments) on the standard BEN assay.

DNA Fragments Added ($\mu\text{g/mL}$)	Fluorescent Signal (\pm SEM)
0	630 \pm 11
0.52	629 \pm 16
1.04	597 \pm 10
2.08	620 \pm 18
4.17	568 \pm 30
8.33	625 \pm 17
16.67	626 \pm 11
33.33	591 \pm 29
66.67	584 \pm 11

Note: Each point was run in replicate (n=6) \pm the standard error of the means (SEM).

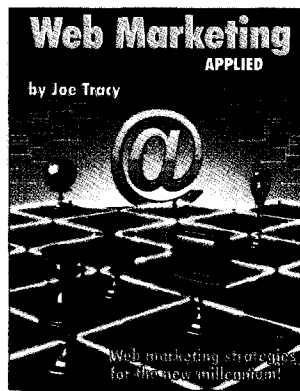
simulate the most rigorous conditions to ascertain that there was no spontaneous breakdown of the beacon substrate.

DNA fragments. DNA fragments were generated by using benzon nuclease to digest calf thymus DNA for 48 hours at 37 °C in assay buffer. Fragments were harvested by passing the sample through a

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1,000 MWCO NanoSep spin filter (Pall Filtron, Northborough, MA). The material was electrophoresed on a 10×20 cm 2% agarose gel for two hours and stained with ethidium bromide to verify complete digestion by comparing the digest to a DNA ladder ranging from 8 to 200 base pairs. The fragments were quantified using an A_{260} measurement, and the purity was ascertained by calculating the A_{260}/A_{280} ratio. Benzonuclease was diluted to 0.0625 U/mL in 150 μ L of assay buffer, and 10 μ L spike volumes of the DNA fragments were added at final concentrations in the assay of 66.7 through 0.52 μ g/mL. After the addition of the 34-mer beacon substrate (beacon 1), the dilution box was incubated overnight and read as previously described.

Substrate stability. Beacon 1 (34-mer) was prepared at 14.5 μ M in PBS, and 0.3 mL aliquots were prepared in Nunc screw-cap CryoTubes (Nalge Nunc International, Rochester, NY). The tubes were wrapped in aluminum foil and incubated at -20°C , 4°C (standard storage condition), or 37°C for the duration of the study. Tubes were sampled weekly and used as substrate in the standard (24-hour substrate incubation) BEN assay for a 0.01 U/mL benzonuclease spike in assay buffer ($n=3$). The tube stored at -20°C was thawed, sampled, and returned to -20°C at each sampling. The

fluorescent signal was averaged for the triplicate samples and plotted for each temperature/time condition.

Synagis spike recovery. Five separate lots of the formulated, lyophilized, humanized monoclonal antibody Synagis were reconstituted in water to a concentration of 100 mg/mL of total protein (25 mM histidine, 1.6 mM glycine, 3% w/v mannitol, pH 6.0), diluted to 1:16 in assay buffer, and spiked with 0.04 U/mL of benzonuclease. The spiked sample was two-fold serially diluted in assay buffer to a final dilution of 1:256. The BEN assay was then performed as described above, and the sample was incubated for 24 hours before analysis on the plate reader. The percent spike recovery was based on the amount of activity calculated from the spiked sample, minus the activity in the unspiked sample, divided by the theoretical spike value.

Substrate incubation. The standard BEN assay (24-hour substrate incubation) was run using each of the three beacon substrates at a 4.45×10^{-7} M concentration. Each assay LLOQ was calculated as described previously.

Modified hyperchromicity. The benzonuclease standard curve was prepared in sample diluent (PBS containing 0.1% w/v of BSA) in a total volume of 0.5 mL per concentration point. Samples were prepared either undiluted or diluted at 1:2 in sample

A LARGE increase in sensitivity was gained by using the molecular beacon as the substrate when compared with the hyperchromicity method.

diluent, and 40 μ L of standard or sample was then transferred to a 1.5 mL microfuge tube. All tubes received additions of 250 μ g of sonicated herring sperm DNA, Tris/magnesium buffer ($[\text{Mg}^{2+}] = 2.5$ mM), 500 μ g of bovine serum albumin (BSA), and benzonuclease spike and were incubated in a 37°C water bath for six hours. The reaction was quenched by adding 0.8 mL of cold 5% v/v perchloric acid to each tube. After vortexing, the tubes were microcentrifuged at 9,000g for five minutes, and 0.4 mL of the supernatant was transferred to a glass tube containing 1.0 mL of PBS. The A_{260} was determined for each sample on a UV spectrophotometer zeroed against PBS. Benzonuclease activity levels were calculated based on a 4-parameter regression to the best fit curve, and percent spike recoveries were calculated based on the theoretical spiking level. Assay acceptability criteria include a correlation coefficient of ≥ 0.995 for the standard curve and spike-recovery levels of 80–120%.

Methyl green. The methyl-green protocol described by Sinicropi involves using salmon sperm DNA dissolved in buffer (25 mM HEPES, 1 mM EDTA, pH 7.5) and stirring for four days at room temperature (RT) at a concentration of 2 mg/mL (8). An aliquot of 11.5 mL of 0.4% crystal violet-free methyl green was added to 192 mL of the dissolved DNA, with the addition of 46 mL of buffer C (25 mM HEPES, 4 mM CaCl_2 , 4 mM MgCl_2 , 0.1% BSA, 0.01% thimerosal, 0.05% Tween-20, pH 7.5). A benzonuclease standard curve and samples were prepared in buffer C, and 100- μ L aliquots were transferred to a 96-well clear microplate. Each well then received an addition of 100 μ L of the DNA substrate,

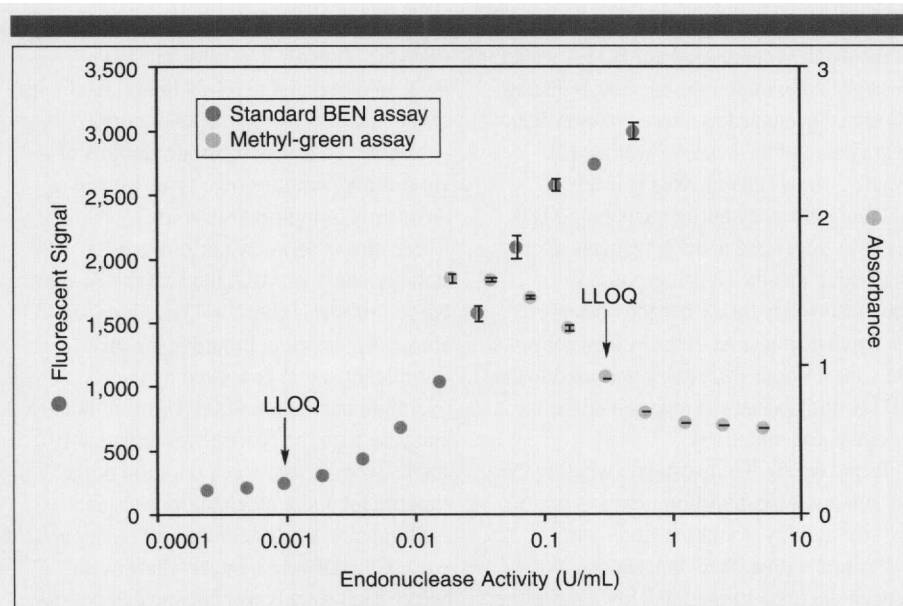


Figure 4. Comparison of the 24-hour incubation time BEN assay with the high-sensitivity version of the methyl-green assay running standard curves prepared with benzonuclease. The BEN assay standard curve points were run in replicates of four, and each point in the methyl-green assay was run in triplicate. Error bars represent the standard error of the means for each point. The arrows denote the LLOQ value for each assay.

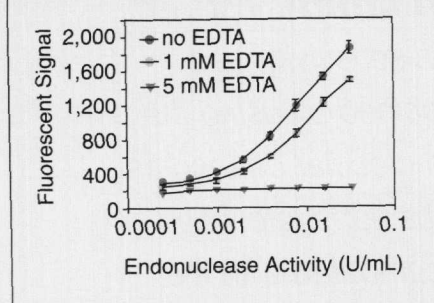


Figure 5. Inhibitory effects of EDTA on benzon nuclease activity in the BEN assay.

was mixed with a multichannel pipette, and then was sealed and incubated for 24 hours at 37 °C. The absorbance was measured at 620 nm, and the nuclease activity was determined from the 4-parameter regressed standard curve. The assay LLOQ was determined by averaging the signal from six replicates (no EDTA, 1 mM EDTA, and 5 mM EDTA), and subtracting 10 standard deviations from that mean signal value. The next higher standard curve point with a coefficient of variation (CV) of $\leq 15\%$ was designated as the assay LLOQ and was determined for each assay plate.

Assay Comparison. Benzon nuclease standard curves were constructed and run in the BEN and methyl-green assays using the protocols above. Each standard curve point was run in triplicate in the methyl-green assay and in quadruplicate in the BEN assay. Six replicates of the blank (no benzon nuclease) were also run in each assay in order to calculate the LLOQ.

Results

Figure 3 shows typical benzon nuclease standard curves comparing the 6- and 24-hour versions of BEN to the modified hyperchromicity assay 3. The LLOQ for both incubation time variations of the BEN assay typically ranges from 0.0005 to 0.002 U/mL, whereas the LLOQ for the modified hyperchromicity method was determined to be 0.7 U/mL based on formal validation of the method. Figure 4 compares the standard curves of benzon nuclease analyzed in the BEN and methyl-green assays with resultant LLOQ values calculated as described previously. The LLOQ for benzon nuclease in the methyl-green assay was 0.3 U/mL, whereas the LLOQ for the BEN assay was 0.001 U/mL.

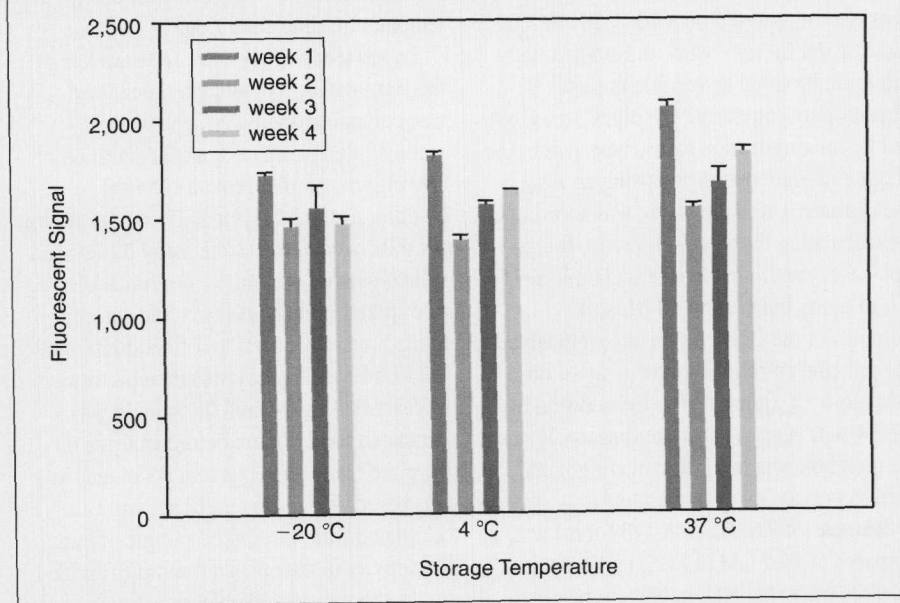


Figure 6. Stability of the beacon reagent as determined by BEN assay performance.

Beacon signal. To better characterize the BEN assay and its limitations, a series of experiments assessed performance parameters. To verify that beacon signal generation in the assay was due to the action of benzon nuclease and not the spontaneous degradation of beacon by other routes, the effect of EDTA on standard curve signal generation was examined. Because the benzon nuclease enzyme has a requirement for Mg^{2+} for enzymatic activity, the addition of the divalent cation chelator EDTA would be expected to inhibit the enzyme, reducing the signal generated as a result of enzymatic degradation of the beacon substrate (2). Figure 5 shows that increasing levels of EDTA in the assay buffer containing Mg^{2+} result in a decrease in the signal generated, suggesting that the signal response is generated solely by the benzon nuclease-mediated degradation of the beacon substrate. Only the 24-hour BEN assay was used in the EDTA study because it represented the most stringent test conditions.

Enzyme activity. To investigate whether the benzon nuclease digestion products inhibit enzyme activity, a spiking study was performed with a fixed concentration of benzon nuclease run in the BEN assay either unspiked or spiked with increasing levels of DNA fragments generated as described previously. Table 1 shows that DNA fragment levels as high as 66.7 $\mu g/mL$ had no inhibitory effect on the enzyme,

indicating that no fragment-induced inhibition of the enzyme took place when compared with the no-fragment-added control. The lack of product inhibition most probably accounts for part of the sensitivity of the assay and the ability to achieve maximal results and sensitivity within six hours of incubation. Shorter incubation times were tried (1–3 hours) but were unacceptable because of the increased variability observed for the standard curve (data not shown). Typically, an assay incubated overnight (16–24 hours) facilitates efficient work flow in the laboratory.

Stability. To assess substrate stability at various temperatures over time, as well as the effects of multiple substrate freeze-thaws on assay performance, a stability study was executed for the 34-mer beacon substrate (beacon 1) as described above. As shown in Figure 6, the mean fluorescent signal generated by the overnight incubation of 0.1 U/mL of benzon nuclease with the beacon was remarkably stable over the four week duration of the experiment under all conditions tested. Additionally, the beacon sample stored at $-20\text{ }^{\circ}\text{C}$ underwent a freeze-thaw cycle before each weekly test but showed no loss of signal-generating potential even at four weeks. These data suggest that the beacon substrate has exceptional stability over a wide range of storage conditions and that the beacon should have an extremely long shelf

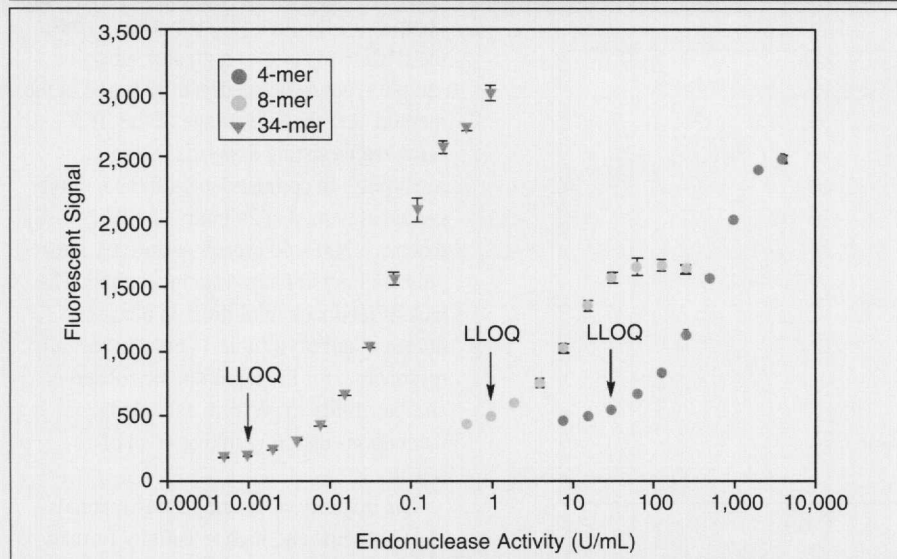


Figure 7. The effects of the size of the loop and stem structures of the beacon substrate on assay performance. All beacons were used at equimolar concentrations (0.445 μ M) and run in the standard BEN assay using benzon nuclease as the endonuclease ($n=3$, mean corrected signal \pm SEM). The arrows denote the calculated LLOQ values for each system using the mean background \pm 10 SD method of LLOQ determination.

life when stored frozen. Additionally, rehydrated beacons stored in the dark at 2–8 $^{\circ}$ C for over one year have been successfully used in the BEN assay (data not shown). For those reasons, the 4 $^{\circ}$ C storage condition was chosen to be optimal for this study, and the variability in signal observed for that condition was considered normal. The test conditions of –20 and 37 $^{\circ}$ C showed no greater variability over the course of the study than that of the beacon stored at 2–8 $^{\circ}$ C.

Sensitivity. Figure 7 shows the results obtained when beacons with variable nucleotide and stem region lengths were used as substrate in the 24-hour BEN assay to quantify benzon nuclease activity. Figure 2 compares the three beacons examined with the bolded nucleotides representing the stem region of the molecule. As shown in that figure, the 34-mer beacon (beacon 1) comprises a 7 base-pair stem region and a 27-nucleotide loop region, whereas the 8-mer beacon (beacon 2) has a 4-nucleotide loop and a 2-nucleotide stem region. Beacon 3 has a 4-nucleotide loop region with no stem region. Because it has been reported by the manufacturer that the end product of DNA digestion with benzon nuclease consists primarily of 2- to 4-mers, it would be expected that the sensitivity of the assay would decrease with decreasing nucleotide

lengths. As Figure 7 shows, the substrate yielding the most sensitive assay was the 34-mer beacon (LLOQ = 0.001 U/mL), followed by the 8-mer (LLOQ = 1 U/mL), and then the 4-mer (LLOQ = 30 U/mL) beacons.

Impurities. Because the BEN assay is homogenous, with no wash or separation steps required, determining the effect of extraneous sample matrix proteins on assay performance is important. To determine the ability of the assay to quantify endonuclease in potentially interfering protein solutions, five lots of a 100 mg/mL concentration of humanized monoclonal antibody (Synagis) were spiked with 0.04 U/mL of benzon nuclease and tested at various twofold serial dilutions in the BEN assay under standard conditions. Table 2 shows the calculated spike-recovery rates for five different lots of the Synagis material. It shows that acceptable spike-recovery (75–125%) levels were obtained for samples diluted at 1:16 or greater, corresponding to a maximal Synagis concentration of 6.25 mg/mL. That particular dilution ratio was selected as a starting point based on previous work (data not shown) that indicated that materials at that concentration (100 mg/mL) required a minimum dilution of 1:16 to obtain acceptable spike-recovery results and therefore to maximize detection sensitivity.

Table 2. Effects of protein interference on benzon nuclease spike-recovery levels in the standard BEN assay.

Lot	1:16	1:32	1:64	1:128	1:256
1	97	84	89	101	112
2	85	82	92	93	106
3	83	78	87	95	103
4	104	94	91	94	92
5	84	100	92	92	95

Note: Dilution values are percentages.

Enzyme source. Figure 8 shows representative standard curves generated in the BEN assay using three commercially available sources of type I endonucleases (Benzonase, Pulmozyme, and OmniCleave). All three endonucleases show a similar LLOQ, which demonstrates that the BEN assay can quantify type I endonuclease activity regardless of the enzyme source. The calculated LLOQ values were 0.00195 U/mL for OmniCleave, 0.000244 U/mL for Pulmozyme, and 0.000975 U/mL for benzon nuclease.

Discussion

A number of methods are reported in the literature to quantify type I endonuclease activity. Our version of the hyperchromicity assay had a determined LLOQ of 0.7 U/mL of benzon nuclease based on formal assay validation studies, which corresponds to about 0.7 ng of enzyme. That assay is undesirable for a number of reasons, but primarily because of its lack of sensitivity, cumbersome manual processing, use of hazardous reagents, and single cuvette reading steps. A commercially available ELISA-based assay kit to quantify residual levels of benzon nuclease, recently introduced by the manufacturer of that product, offers a significant improvement in sample throughput without the use of hazardous chemicals. The claimed sensitivity of that assay is 0.5 ng/mL (0.5 U/mL), which offers only a modest increase in sensitivity when compared with the hyperchromicity method. Moreover, a severe limitation of the ELISA assay is that because a benzon nuclease-specific antibody is used, only benzon nuclease can be quantified. Thus, from a production standpoint, if that source of endonuclease became unavailable, a new assay would need to be put in place to measure the new

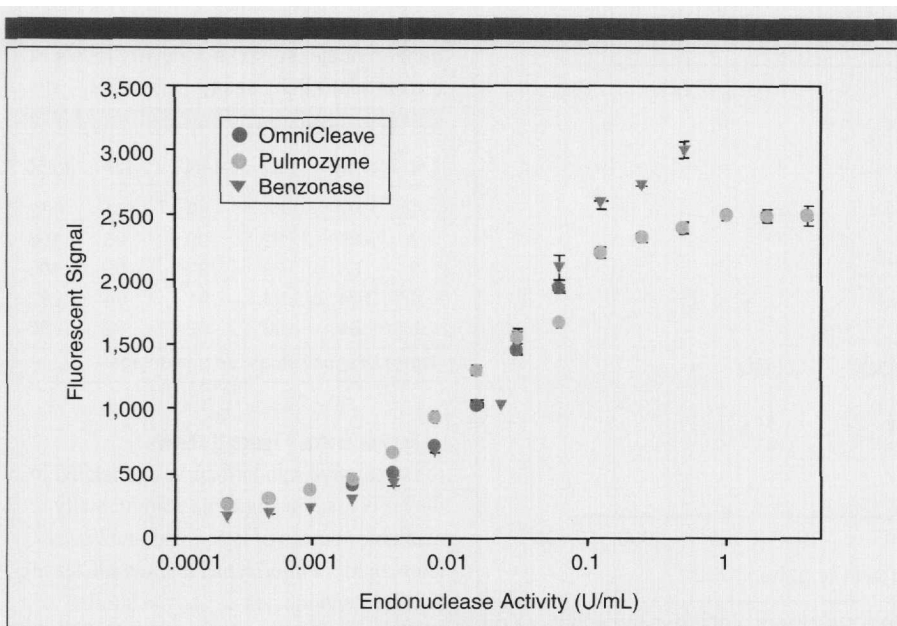


Figure 8. Results from the standard BEN assay showing that the assay, using the 34-mer substrate beacon, has the ability to quantify type I endonuclease activity from a variety of commercial sources for the enzyme. Each standard curve point was run in triplicate (\pm standard error of the means) in the assay.

endonuclease level. Of more importance, the ELISA format lacks enzyme activity data, which is crucial to address the safety concerns surrounding low levels of endonuclease present in final products.

A recent article described a 96-well plate-based homogenous assay to quantify enzyme activity in the type I endonuclease Pulmozyme (8). This method has the advantage of being homogenous (requiring no wash or separation steps), unlike the previous hyperchromicity and ELISA methods. In our tests, we obtained greater sensitivity with this method than with the hyperchromicity method, but the sample matrix interference made the assay unusable as a trace residual assay. Also, the preparation of the DNA-dye substrate takes three to four days, and the final substrate has a storage life at 4 °C of less than 30 days. Several drawbacks to the methyl-green assay method center on the physical properties of the dye-DNA complex. The authors reported that the dye-DNA complex was less stable in certain buffers, such as Tris, and that phosphate buffer was a potent inhibitor of the methyl-green assay.

In our experiments to develop an assay with improved sensitivity for endonuclease activity, our initial studies used a 34-mer beacon with the loop region using the sequence of the mouse β -actin gene. We chose that sequence because it was well

characterized by the company that synthesized the beacon reagents and because of the sequence-independence of type I endonucleases. Our initial experiments centered solely on benzon nuclease, and the BEN assay was characterized primarily using that nuclease. A large increase in sensitivity (nearly 1,400-fold) was gained by using the molecular beacon as a substrate when compared with the modified hyperchromicity method (Figure 3). Also, only modest sample dilutions needed to be performed to eliminate sample matrix interference with the assay, based on the spike-recovery studies (Table 2).

The 34-mer beacon substrate (beacon 1) demonstrated exceptional stability in the tests we performed. The EDTA studies showed that the signal generated in the assay was due to specific degradation of the substrate by benzon nuclease, and not by spontaneous breakdown of the beacon over the course of the assay. The beacon showed exceptional stability at a number of temperatures, even after four freeze-thaw cycles (Figure 6). Because of that stability, large amounts of beacon can be obtained and stored frozen for an extended period of time, yielding consistent results in the assay. Because the BEN assay uses covalently coupled fluorophore and quench on the nucleotide structure, the substrate is relatively resistant to buffer and pH changes

compared with the noncovalent dye-DNA complex of the methyl-green assay. Thus, the BEN assay allows a greater subset of sample types to be successfully tested in the method. Another advantage of the BEN assay over existing assays is its high sensitivity. In comparison with the methyl-green assay read at 24 hours, the BEN assay reaches a 300-fold greater sensitivity after only six hours of substrate incubation. The lack of feedback inhibition on benzon nuclease activity (Table 1) may be partially responsible for the rapid kinetics observed and the ability to perform extended incubations resulting in that level of sensitivity.

The majority of existing assay methods have both low and high sensitivity formats so that an assay can be tailored to the type of samples being analyzed. Increased sensitivity is usually achieved by increasing the incubation time of the sample with the substrate. Our studies concluded that varying the size of the beacon substrate could modulate assay sensitivity independent of the incubation time. Studies performed with beacons of different sizes and stem region composition were compared with the 34-mer standard beacon. Substrates of similar size to the theoretical size of the final digest fragment size (2- to 4-mers) would be expected to be poor substrates for benzon nuclease and would result in decreased assay sensitivity. The substitution of an equimolar amount of a 4-mer with no stem region (beacon 3) for the standard 34-mer showed a 30,000-fold decrease in assay LLOQ in the 24-hour BEN assay when compared with the 34-mer substrate (Figure 7). Interestingly, the use of an equimolar amount of an 8-mer with a 2-nucleotide stem region (beacon 2) gave the expected intermediate assay sensitivity, but the maximal fluorescent intensity was significantly reduced when compared with the 34-mer and 4-mer substrates (Figure 7). We hypothesized that perhaps the nuclease could not efficiently digest the 2-nucleotide stem region or that dimerization of the beacons was occurring that resulted in a mixed population of substrate molecules. Experiments using the 34-mer beacon to determine the role of the stem and loop regions in signal generation by endonuclease used BAL31 and mung bean nucleases. They failed because of the nonspecific nature of those nucleases for single rather than double-stranded DNA

(data not shown). We did not examine beacon substrates larger than 34 nucleotides long because of the cost associated with their synthesis and because we did not need further sensitivity.

One of the advantages of an activity-based assay is that theoretically any type I nuclease activity could be quantitated with minimal changes to the assay. We demonstrated this by running standard curves in the standard BEN assay at the same activity levels with the commercially available endonucleases Benzonase, Pulmozyme, and OmniCleave (Figure 8). The curves could be nearly superimposed, indicating that the BEN assay could be used to quantify activity levels for any of those commercial preparations. Optimal assay performance was observed with the benzon nuclease standard curve because the dynamic range of the curve's linear portion was broader than either of the other nucleases. Presumably, that was because the BEN assay buffer was designed for the optimal performance of benzon nuclease based on its pH and ionic requirements. We

hypothesize that the Pulmozyme and OmniCleave curves could be optimized in the BEN assay by changing the formulation of the assay buffer. Despite that shortcoming, the LLOQ values were within the typical range observed for benzon nuclease (Figure 8).

Perhaps, molecular beacon technology could be exploited as a rapid method for determining enzyme activity of sequence-specific restriction endonucleases (type III). The stem region, which is the only double-stranded portion of the beacon, could be synthesized with the recognition sequence for a particular restriction enzyme. Incubation with that enzyme under the proper buffer conditions may result in cleavage and subsequent signal generation. That would allow development of a rapid homogenous assay to measure restriction enzyme activity in commercial restriction enzyme products.

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