MERCK MILLIPORE

Benzonase® endonuclease

Balancing efficiency and regulatory compliance – the smart solution for DNA removal in biopharmaceutical production

Merck Millipore is a Division of MERCK

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Benzonase® endonuclease

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Perfectly poised for compliance and higher yields

Meeting the strictest possible purity standards for biopharmaceuticals is a challenge all its own. Want to raise the bar even higher? Increase your downstream processing yields at the same time. Fortunately, there's Benzonase® endonuclease from Merck Millipore. It's the one effective method to remove both DNA and RNA, whether you're in the lab or working with industrial-scale processes.

A unique, genetically engineered endonuclease, Benzonase® endonuclease offers a variety of advantages over existing methods of nucleic acid removal. You'll see it at work already in various applications throughout the pharmaceutical and biotechnological industry. And Benzonase® endonuclease has proven its value in the lab for over 20 years.

Additional Merck Millipore products for your biopharmaceutical applications: amino acids; high-quality mineral salts and buffers; Fractogel® EMD bioprocessing resins; silica gels; caustic agents; magnesium chloride, Pellicon® 2 and Pellicon® 3 filters; Millistak+® product line. For more information please visit www.merckmillipore.com

Benzonase® endonuclease also offers you the reliability and high quality you've come to expect from us – because it's manufactured under GMP conditions and exclusively distributed by Merck Millipore and our associates. We invite you to discover how Benzonase® endonuclease helps you work more efficiently.

2 About Benzonase® endonuclease

What's in Benzonase® endonuclease for you?

- the capability to attack and degrade all forms of DNA and RNA
- the absence of detectable proteolytic activity
- a wide range of operating conditions
- the advantages of exceptionally high specific activity
- the assurance that you'll meet industry requirements for reliable supply and consistent high quality thanks to GMP manufacturing
- the support by our EMPROVE® bio dossier and a DMF type II file (FDA Reg. No. BBMF 5403; current version 2013, available in eCTD format)
- the availability in bulk quantities for the use in R&D up to manufacturing scale
- a guideline on its use and its removal from biopharmaceutical processes

Benzonase® endonuclease has been especially designed for applications in biotechnological processing, such as:

- removing DNA/RNA from proteins and other biologicals
- reducing viscosity caused by nucleic acids
- preparing samples in electrophoresis and chromatography
- preventing cell clumping

Why Benzonase® endonuclease is the right choice

When you choose Benzonase® endonuclease, you're choosing an endonuclease that ensures exceptionally high purity and activity for your finished product. This enzyme has been designed to be supplied without protease activity and without the viral contaminants that can accompany enzymes isolated from natural sources. To meet the widest possible range of processing and cost requirements, Benzonase® endonuclease is available in different grades of purity:

- **Benzonase® endonuclease, purity grade I (≥99%) EMPROVE® bio**
- **Benzonase® endonuclease, purity grade II (≥90%)**

Benzonase® endonuclease is a genetically engineered endonuclease produced in *Escherichia coli (E. coli)* strain W3110, a mutant of strain K12, which contains the proprietary pNUC1 production plasmid. This plasmid encodes an endonuclease normally expressed in *Serratia marcescens.*

Meet the highest standard

U.S. FDA regulations are regarded as one of the world's strictest quality and purity standards for biopharmaceuticals. To date, the U.S. FDA has not issued any explicit regulations governing the production and use of process materials such as Benzonase® endonuclease. Although they do not appear on the final dosage form, these process materials are used in the direct purification of active pharmaceutical ingredients.

In other words, finished biopharmaceutical products that are safe call for process materials that are safe, too. At Merck Millipore, we never compromise on safety. That's why Benzonase® endonuclease is manufactured under GMP conditions. The U.S. FDA also stocks a DMF type II file for Benzonase® endonuclease (Reg. No. BBMF 5403; current version 2013), available in eCTD format. We also ensure quality throughout our organization with a quality management system in accordance with DIN ISO 9001 and 14001.

A complete package

The finished product does not contain additives of animal origin, such as stabilizers like bovine serum, albumin, or gelatin. Benzonase® endonuclease is supplied in 50% glycerol solution to prevent the preparation from freezing. The glycerol is of synthetic origin. During production of Benzonase® endonuclease, the fermentation medium contains casamino acids from bovine milk. This milk is considered fit for human consumption. Please don't hesitate to ask us for full batch documentation that meets U.S. FDA requirements as of January 1, 2011.

Viral safety

Benzonase® endonuclease is prepared using a well-defined bacterial expression system (*E.coli* strain W3110). The risk of viral contamination is regarded as negligible.

Microbiological safety

Each batch of Benzonase® endonuclease, purity grade I and II containing 100,000 or more enzyme units is tested using a modified EP method to ensure it only contains trace levels (<10 CFU in 100,000 U) of aerobic bacteria, yeasts, and molds.

Endotoxin testing

Each batch of Benzonase® endonuclease, purity grade I containing 100,000 or more enzyme units is tested for endotoxins using the well-known LAL test. The total endotoxin level is below 0.25 EU per 1,000 units.

Product quality assurance:

Using Benzonase® endonuclease to remove DNA/RNA

U.S. FDA guidelines for the manufacture of recombinant biologicals for therapeutic use demand that nucleic acid contamination should be limited to 100 pg per dose (in the end product). Benzonase® endonuclease, when used under appropriate reaction conditions, will degrade all nucleic acid sequences down to oligonucleotides of approximately three to five base pairs in length – which is significantly below the hybridization limit – enabling recombinant proteins to meet the FDA guidelines for nucleic acid contamination.

Cell disintegration:

Using Benzonase® endonuclease to reduce viscosity

The ability of Benzonase® endonuclease to rapidly hydrolyze nucleic acids makes the enzyme an ideal choice for reducing cell lysate viscosity – both in the research laboratory and the manufacturing plant.

Using Benzonase® endonuclease to reduce viscosity also allows you to:

- reduce processing time
- increase the yield of protein products
- improve the separation of pellets and supernatants in centrifugations
- facilitate the filtration of solutions, especially ultrafiltration
- increase the efficiency of chromatographic purification steps (e.g., in expanded bed adsorption)
- boost the productivity of cross-flow microfiltration steps (e.g., in inclusion body processing)

Benzonase® endonuclease may be used with all methods of cell lysis, including lysozyme treatment, freeze-thawing procedures, and high-pressure homogenization. Although Benzonase® endonuclease may be added post lysis, it was shown that, when the same amount of Benzonase® endonuclease is added before lysis instead of afterwards:

- the amount of Benzonase® endonuclease required for nucleic acid hydrolysis can be reduced 50 to 200 times
- viscosity reduction will occur significantly faster

Particle processing:

Using Benzonase® endonuclease to facilitate particle purification

It is well known that nucleic acids may adhere to cell-derived particles such as viruses or inclusion bodies. This adhesion may interfere with separation due to agglomeration, change in particle size or change in particle charge, resulting in a reduced product yield. Benzonase® endonuclease is well suited for reducing the nucleic acid load during purification, thus eliminating interferences and improving both yield and purity of the end product.

Bioanalytical applications:

Using Benzonase® endonuclease for sample preparation

Bioanalytical applications of Benzonase® endonuclease include sample preparation for e.g. ELISA, chromatography or two-dimensional electrophoresis (protein mapping), and footprint analysis. Benefits of sample treatment include improved resolution and increased recovery of samples.

CERTIFICATE
DQS GmbH
hereby certifies that the company
Merck KGaA
Frankfurter Straße 250
D-64271 Darmstadt
Mainzer Straße 41
D-64579 Gernsheim
with the organizational units/sites as listed in the annex
for the scope
Development, manufacture and distribution of basic chemicals, special chemical products and analyzer systems
has implemented and maintains a
Quality Management System.
An audit, documented in a report, has verified that this quality management system fulfills the requirements of the following standard:
DIN EN ISO 9001 : 2000
December 2000 edition
This certificate is valid until 2005-11-17
Certificate Registration No.: 005356 QM
Frankfurt am Main, Berlin 2002-11-18
Dr.-Ing. K. Petrick
MANAGING DIRECTORS
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D-60433 Frankfurt am Main, August-Schanz-Straße 21
D-10787 Berlin, Burggrafenstraße 6
Deutscher Akkreditierungs Rat
DAR
TGA-ZM-02-90-00
TGA-ZM-02-90-60

3 Enzyme Characteristics

Benzonase® endonuclease is designed to help your products meet the world's most exacting standards for biopharmaceuticals. So it's to your advantage to know exactly what makes Benzonase® endonuclease work the way it does.

General profile

Benzonase® endonuclease is a protein consisting of two subunits with a molecular weight of about 30 kD each. The protein has an isoelectric point (pI) at pH 6.85. It is functional between pH 6 and 10, and from 0 °C to above 42 °C. Mg^{2+} (1–2 mM) is required for enzyme activity.

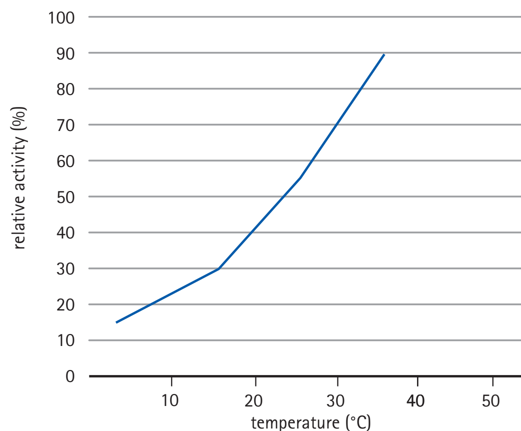


Figure 1.
Effect of temperature on Benzonase® endonuclease activity

Substrate specificity

Benzonase® endonuclease acts as an endonuclease that degrades both of DNA and RNA - whether single-stranded, double-stranded, linear, circular, or supercoiled. No base preference is observed. As with all endonucleases, Benzonase® endonuclease hydrolyzes internal phosphodiester bonds present between the nucleotides. Upon complete digestion, all free nucleic acids present in solution are reduced to 5'-monophosphate-terminated oligonucleotides which are three to five bases in length.

Activity

Specific activity for Benzonase® endonuclease is measured under standard assay conditions (see Table 1, page 8). Minimum specific activity for Benzonase® endonuclease, purity grade II (≥90%) is 1.0 x 10^6 units/mg protein. Minimum specific activity for Benzonase® endonuclease, purity grade I (≥99%) is 1.1 x 10^6 units/mg protein.

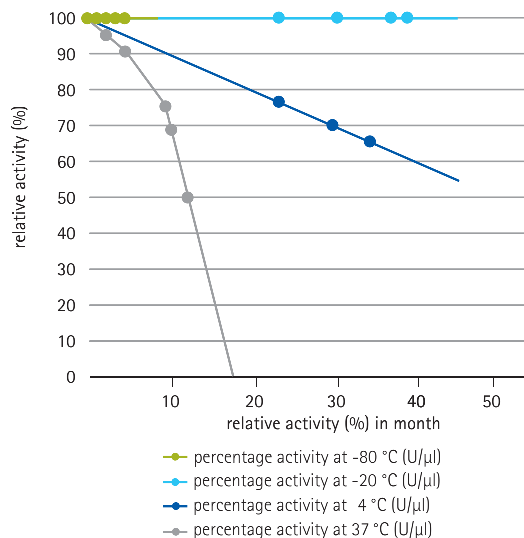


Figure 2a.
Effect of various storage temperatures on Benzonase® endonuclease activity over time

Temperature stability of Benzonase® endonuclease

The optimum temperature for the degradation of nucleic acids by Benzonase® endonuclease is 37 °C. The enzyme is, however, effective over a temperature range of 0–42 °C (see Fig. 1). The optimum storage temperature is -20 °C to prevent loss of activity or freezing. The effect of storage at various temperatures is illustrated in Fig. 2a. Fig. 2b shows the Benzonase® endonuclease stability at 25 °C (60% RH). We do not recommend repeated freeze/thaw cycles and storage at temperatures lower than -20 °C.

Product purity

Analysis of Benzonase® endonuclease, purity grade II (≥90%) by SDS-PAGE results in a dominant band corresponding to Benzonase® endonuclease. All other proteins present (<10%) are derived entirely from *E.coli*. Benzonase® endonuclease, purity grade I (≥99%) EMPROVE® bio is produced by chromatographic purification of Benzonase® endonuclease, purity grade II. Analysis of Benzonase® endonuclease, purity grade I by SDS-PAGE results in a single band corresponding to Benzonase® endonuclease. All other proteins present (<1%) are derived entirely from *E.coli*. The preparation does not contain any antimicrobial preservatives or protein stabilizers except glycerol (of synthetic origin). The solution has been filtered through a 0.2 µm filter.

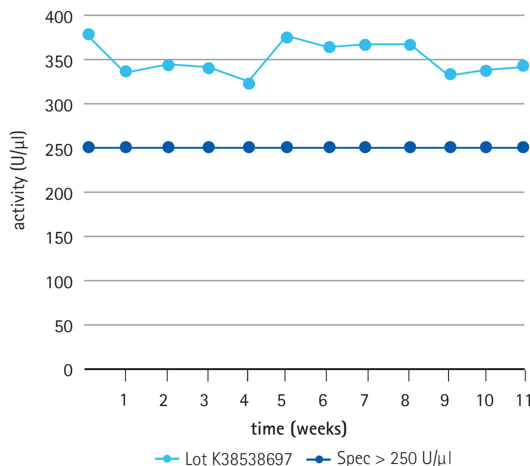


Figure 2b.
Stability of Benzonase® endonuclease stored at 25 °C/60% RH

Benzonase® endonuclease was stored in simulated primary package material at 25 °C/60% RH. The activity of lot K38538697 was measured once per week according to the monograph. After a storage time of 11 weeks the activity remained unchanged.

Unit definition

A standard assay was developed to define the activity of Benzonase® endonuclease. The procedure is based on the measurement of changes in optical density that occur when oligonucleotides are released into solution during digestion of DNA with Benzonase® endonuclease. The assay is performed using excess substrate but other conditions are optimum. The rate of DNA degradation is measured by precipitation of undigested DNA using perchloric acid. Based on this assay, one unit of Benzonase® endonuclease is defined as the amount of enzyme that causes a change in absorbance at 260 nm of 1.0 absorption units within 30 minutes. One unit of Benzonase® endonuclease also corresponds approximately to the amount of enzyme required to completely digest 37 µg of DNA in 30 minutes under standard assay conditions.

For a detailed description of the standard assay see Appendix.

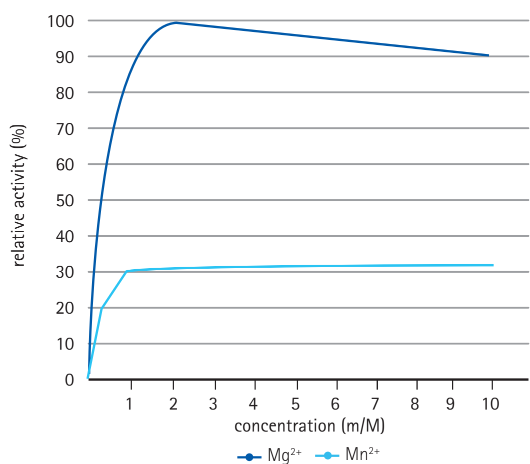


Figure 3.
Effect of magnesium and manganese ion concentrations on Benzonase® endonuclease activity

A concentration of 1 to 2 mM Mg^{2+} or Mn^{2+} is essential for activity of Benzonase® endonuclease. Mg^{2+} is preferred because it enables the enzyme to reach its optimal level of activity. Ca^{2+} and Sr^{2+} do not have effects on the activity of the enzyme.

Operating conditions

Benzonase® endonuclease retains its activity under a wide range of operating conditions, as specified in Table 1 and Fig. 3 to 6. Benzonase® endonuclease is active in the presence of ionic and non-ionic detergents, urea, and ammonium sulfate. This is illustrated in Fig. 7 to 10.

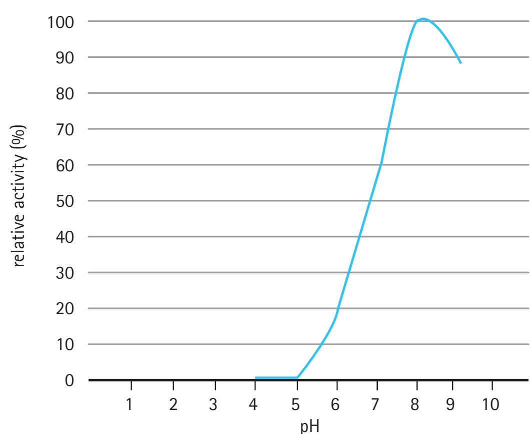


Figure 4.
Effect of pH on Benzonase® endonuclease activity

The incubation buffers are 20 mM Tris, 20 mM histidine and 20 mM MES. Although not shown here, a slight buffer effect has been observed, indicating minimal changes in activity due to the effects of different buffers.

Table 1.
Benzonase® endonuclease reaction conditions

Condition	Optimal*	Effective**
Mg^{2+}	1–2 mM	1–10 mM
pH	8.0–9.2	6.0–10.0
Temperature	37 °C	0–42 °C
Dithiothreitol (DTT)	0–100 mM	>100 mM
ß-Mercaptoethanol	0–100 mM	>100 mM
Monovalent cation concentration (Na^+, K^+, etc.)	0–20 mM	0–150 mM
PO_4^{3-} concentration	0–10 mM	0–100 mM

** "Optimal" is defined as the condition under which Benzonase® endonuclease retains ≥90% of its activity.*
*** "Effective" is defined as the condition under which Benzonase® endonuclease retains >15% of its activity.*

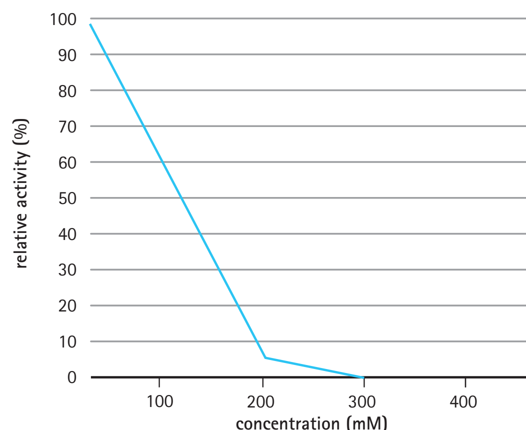


Figure 5.
Effect of monovalent cations on Benzonase® endonuclease activity

The enzyme exhibits an identical response to Na^+ and K^+, presumed that all other monovalent cations have a similar effect.

Protease activity

Benzonase® endonuclease is free of detectable protease activity, making the enzyme ideal for production processes in which high yields of biologically active proteins are desired. The absence of proteolytic actvities is monitored by a highly sensitive and validated assay using a resorufin-labeled casein.

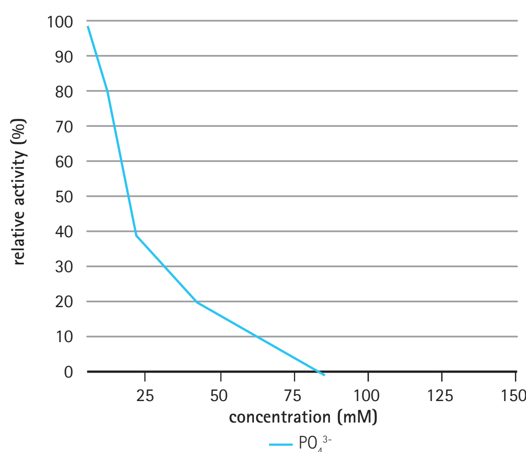


Figure 6.
Effect of phosphate ion concentration on Benzonase® endonuclease activity

The incubation buffer is a Tris-phosphate buffer.

Effect of guanidine HCl, EDTA, and PMSF on Benzonase® endonuclease activity

Using the standard assay for Benzonase® endonuclease activity, it was shown that concentrations of guanidine HCl exceeding 100 mM completely inhibit the enzyme activity. An EDTA concentration of 1 mM partially inhibits Benzonase® endonuclease. However, a concentration of 5 mM EDTA causes a >90% loss of enzyme activity by complexing the essential Mg^{2+} ions. PMSF in a concentration of 1 mM does not inhibit Benzonase® endonuclease.

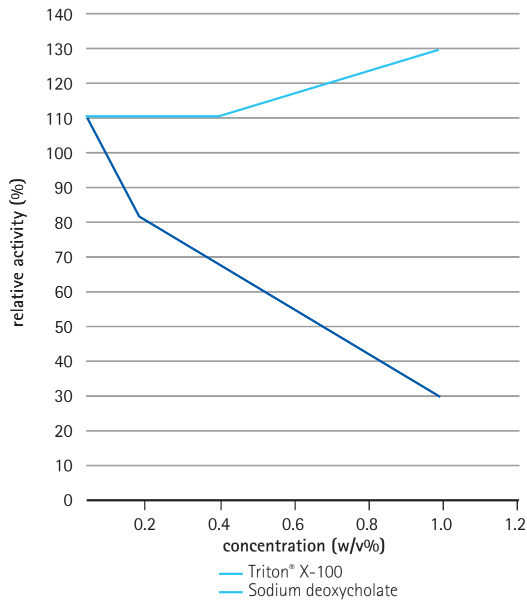
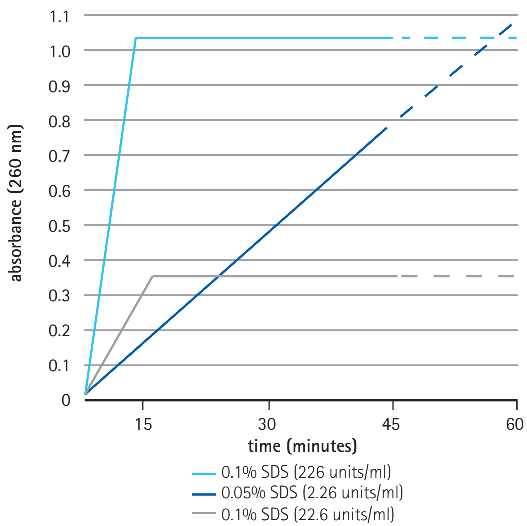


Figure 7.

Effect of detergents (Triton® X-100, sodium deoxycholate) on Benzonase® endonuclease activity

- Concentrations of Triton® X-100 <0.4% have no effect on the activity of Benzonase® endonuclease.
- At concentrations of sodium deoxycholate <0.4%, Benzonase® endonuclease retains at least 70% of its activity.



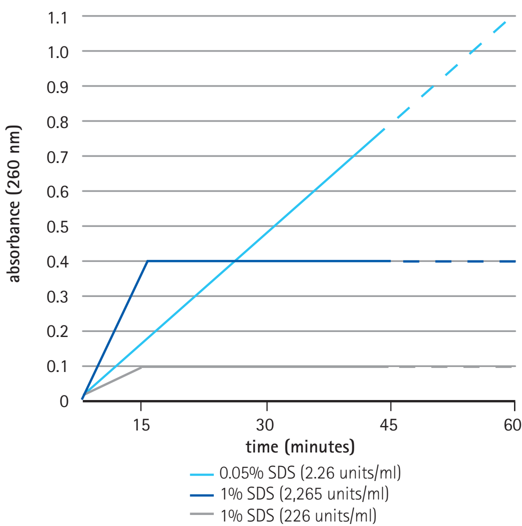


Figure 8a and 8b.

Effect of different sodium dodecyl sulfate (SDS) concentrations on Benzonase® endonuclease activity

- Benzonase® endonuclease retains 100% of its activity in sodium dodecyl sulfate (SDS) concentrations up to 0.05%.
- At SDS concentrations between 0.1% and 1%, Benzonase® endonuclease remains active for a short period of time before being denatured, this is illustrated by the horizontal portions of the graphs. This can be partially compensated by increasing the concentration of Benzonase® endonuclease.

Figure 9a.
Effect of different concentrations of urea on Benzonase® endonuclease activity

- Benzonase® endonuclease is activated by urea at concentrations up to approximately 6 M.
- At 6 M urea, enzyme activity first increases, then decreases over time.
- At 7 M urea, Benzonase® endonuclease denatures after 15 minutes, and activity is lost. However, significant degradation of nucleic acids occurs before the enzyme is inactivated.

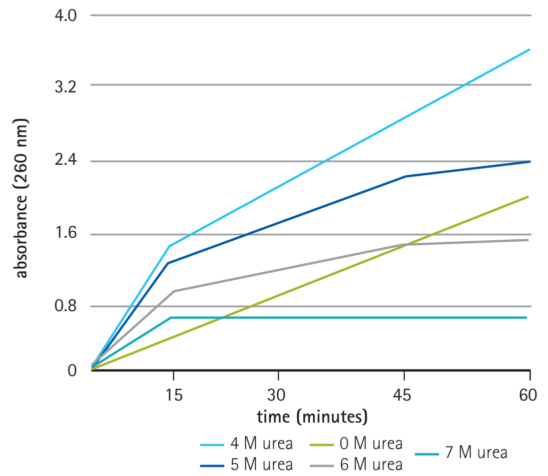


Figure 9b.
Compensatory effect of different Benzonase® endonuclease concentrations at constant urea concentration

Higher concentrations of Benzonase® endonuclease can partially compensate the effects of 7 M urea.

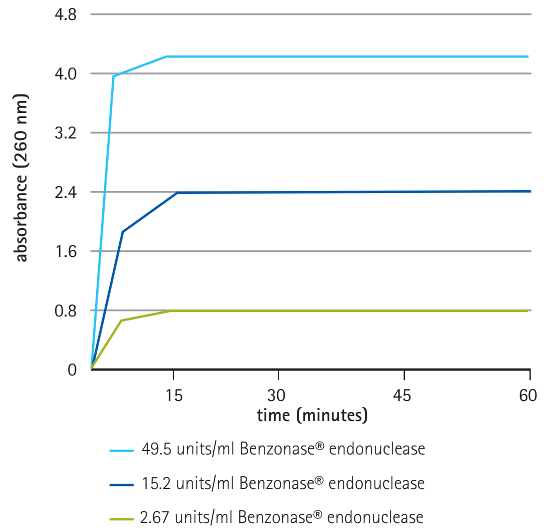
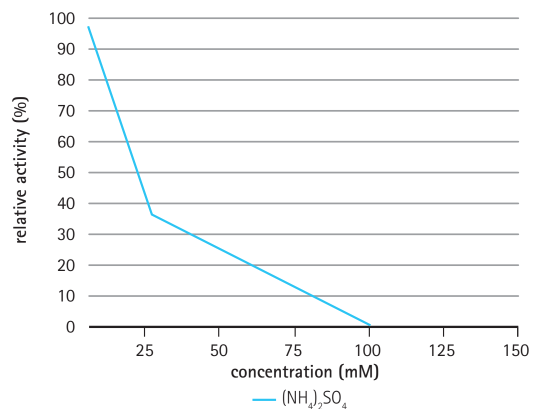


Figure 10.
Effect of ammonium sulfate on Benzonase® endonuclease activity

Benzonase® endonuclease is inhibited by higher concentrations of ammonium sulfate but remains active at concentrations <100 mM.



Typical Applications

4

One of the great things about Benzonase® endonuclease is how many different kinds of applications you can use it in. Over the pages that follow, you'll find a guide to some examples.

Although these examples do not constitute guidance on the use of Benzonase® endonuclease, they provide general information on how to address specific challenges involving residual nucleic acids in biopharmaceutical processes.

Why choose Benzonase® endonuclease over DNAse or RNAse?

- It possesses a higher specific activity
- It is free of detectable proteolytic activities
- It is the only endonuclease produced in bulk quantities under GMP conditions for large-scale use
- It has been especially designed for application in biotechnological processing and biopharmaceutical production
- It can be used effectively in various fields of applications
- It has a proven track record in various commercial vaccine manufacturing processes

Example 1: Elimination of nucleic acids from recombinant proteins

Operating conditions

Recombinant biopharmaceuticals must meet strict requirements concerning residual nucleic acids. This is a typical field of application for Benzonase® endonuclease, ensuring compliance with growing regulatory requirements. To demonstrate the efficiency of Benzonase® endonuclease in DNA fragment elimination, an experiment was designed using a high burden of DNA (50 µg/ml). Hence it can be regarded as a worst-case scenario, since the DNA content usually found in an operational bioprocessing environment is likely to be significantly lower. Indeed, the typical concentration of DNA encountered in bioprocesses lies between 0.5 and 5.0 µg/ml. The following experiment illustrates the influence of incubation time, temperature, and enzyme concentration on enzyme activity.

Experimental design

A solution of herring sperm DNA with a final concentration of 50 µg/ml was made up using standard test buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 0.1 mg/ml BSA, pH 8.0). The progress of the reaction was assayed as follows: At different time intervals (0–30 hours) aliquots of 10 µl (initially containing 500 ng of DNA) were applied to a nitrocellulose membrane and hybridized with a ^{32}P-labeled probe of nick-translated herring sperm DNA. Hybridization and washing steps were carried out under low stringency conditions in order to optimize the detection of repeated sequences in the herring sperm DNA. After washing, the filter was subjected to autoradiography for one to thirty hours using an amplifying screen. DNA standards from 100 ng to 10 pg allowed a semiquantative evaluation of the residual hybridizable DNA.

Results

Reducing the enzyme concentration while keeping all other parameters constant results in a longer incubation time. A reduction of the enzyme concentration has less influence on the initial rate than on the total time required to reach the 10 pg level. After four hours at a concentration of 90 U/ml, 99.95% of the DNA can no longer be hybridized (see below). However reducing the initial enzyme concentration by 90%, i.e. to 9 U/ml, still yields 99% of the DNA as non-hybridizable (see Table 2).

Table 2.

	Residual hybridizable DNA (in pg) after incubation for			
Benzonase® endonuclease concentration	0 h	4 h	6 h	22 h
90 U/ml	500,000	200	20	n. d.
9 U/ml	500,000	5,000	2,000	300

DNA concentration: 500 ng per sample – Temperature: 37 °C – n. d. = not detectable (<10 pg)

Decreasing the incubation temperature results in increased incubation times (see Table 3).

Table 3.

	Residual hybridizable DNA (in pg) after incubation for				
Incubation temperature	0 h	4 h	6 h	22 h	30 h
37 °C	500,000	200	20	n. d.	n. d.
23 °C	500,000	500	100	10	n. d.
0 °C	500,000	1,000	500	50	10

DNA concentration: 500 ng per sample – Benzonase® endonuclease concentration: 90 units/ml – n. d. = not detectable (<10 pg)

Since PBS is commonly used in bioprocesses, the activity of Benzonase® endonuclease in PBS compared to that in Tris was determined (see Table 4).

Table 4.

	Residual hybridizable DNA (in pg) after incubation for				
Buffer	0 h	4 h	6 h	22 h	30 h
Tris buffer	500,000	500	100	n. d.	n. d.
PBS buffer	500,000	5,000	1,000	500	300

DNA concentration: 500 ng per sample – Benzonase® endonuclease concentration: 90 units/ml – Temperature: 23 °C

The experiment shows that complete DNA fragment elimination can be achieved using Benzonase® endonuclease. Due to its high specific activity (1 x 10^6 units/mg protein) it is sufficient to add the enzyme in negligible concentrations (10–100 ng/ml), even under reaction conditions far from the optimum. In conclusion it can be said, that an optimization of the conditions should be based on reaction time, temperature, and concentration. Note that the three factors influence each other (see page 16, Benzonase® endonuclease triangle).

Example 2: Viscosity reduction I

When purifying proteins, the first step is to disrupt the cells or tissue sample and extract the relevant protein fraction. The purification process should be carried out without any time delay in order to minimize losses of the target protein by proteolytic attack.

However, cell extracts often show high viscosity due to the release of nucleic acids during disintegration of cells. As a result subsequent purification will be impeded. The first separation steps should be carried out without any time delay in order to mimimize target protein loss. The addition of Benzonase® endonuclease rapidly hydrolyzes nucleic acids resulting in viscosity reduction thus reducing processing times and increasing protein yields.

Experimental design

Two 100 µl aliquots of a human granulocyte preparation (1.0×10^8 cells/ml, ca. 5 mg of total protein) were removed and centrifuged. The cells were collected and resuspended in 50 µl of a 20 mM Tris-HCl pH 9.0 buffer containing 7 M urea, 100 mM DTT, and 1% Triton® X-100. 2.5 units of Benzonase® endonuclease, purity grade I (≥99%) were added to sample b (see Fig. 11). This corresponds to a final concentration of 50 units/ml. All samples were incubated at 4 °C for 5 minutes and then centrifuged at 10,000 x g for 1 minute. This experiment was repeated using two 1 ml aliquots.

Results

After centrifugation the samples without Benzonase® endonuclease (a and c) retain a high viscosity, with no clear demarcation between supernatant and pellet (see Fig. 11). However, the samples containing Benzonase® endonuclease (b and d) show a large reduction in viscosity. A dense pellet is formed which allows easy removal of the supernatant. The recovery of protein was significantly increased when the sample had been treated with Benzonase® endonuclease. This can be explained by the fact that certain proteins tend to be trapped in the cell debris and viscous supernatant containing nucleic acids. During upscaling the negative impact of high viscosity becomes even more obvious.

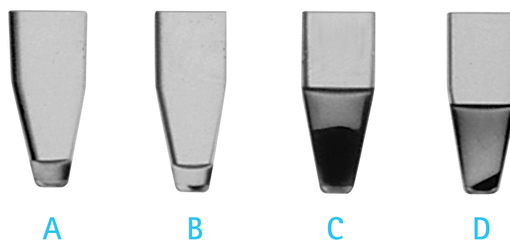


Figure 11.
Protein extracts of human granulocytes: **a)** without Benzonase® endonuclease, **b)** with Benzonase® endonuclease (5 units); **c), d)** same condition as in **a), b),** but volumes 10 times increased.

Example 3: Viscosity reduction II

Experimental design

7.5 g of *E.coli* W3110 (wet weight) were suspended in 15 ml of 10 mM Tris-HCl buffer pH 9.0, 1 mM EDTA. $MgCl_2$ was added to obtain a final concentration of 6 mM. Five 3 ml samples were taken from this suspension. Benzonase® endonuclease, purity grade II (≥90%) was added to each of these aliquots yielding a concentration gradient. The aliquots were passed through a French press at 10,000 psi and immediately incubated at 0 °C. Changes in viscosity were visually followed by measuring viscoelastic properties with a pipette. The time of release from the press was taken as start of the reaction (t = 0).

Table 5.

Sample	Benzonase® endonuclease	Time required to obtain "aqueous" drops
1	0.24 U/ml	>60 min
2	2.40 U/ml	15 min
3	8.00 U/ml	5 min
4	24.00 U/ml	1.25 min
5	240.00 U/ml	0 min

Results

Normally, the extracts from the French press are released as highly viscous drops. As the amount of Benzonase® endonuclease increases, the time required to obtain "aqueous" drops decreases.

Example 4: Purification of protein fragments from inclusion bodies

Inclusion bodies can be an attractive alternative method for the production of overexpressed proteins in *E.coli*. High product yields are often possible; they are also extremely resistant to external influences and, due to their high densities, a simple but effective mechanical purification procedure is possible. However, for successful renaturation of the solubilized inclusion bodies, any adhering proteases must be completely removed. This can be impeded by the presence of large quantities of DNA in the bacterial lysate. The problem can be solved by using a combination of mechanical DNA homogenization (ultrasound) and enzymatic hydrolysis with Benzonase® endonuclease. Using this procedure, the production and purification of proteins and protein fragments otherwise sensitive to proteolytic attack becomes practicable. This is illustrated by the following example. Two fragments (N- and C-termini) of mitochondrial creatine kinase (Mi-CK) were biotechnologically produced in *E.coli*. Both fragments were successfully produced in large quantities by the bacteria and accumulated in the form of inclusion bodies. However, unlike the wild-type Mi-CK, the soluble form of the fragments is highly sensitive to proteolytic activity, a fact that rendered complete removal of any adhering proteases a prerequisite for successful purification of the renatured proteins.

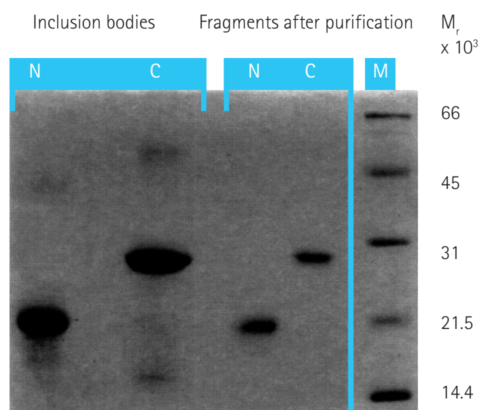


Figure 12.
SDS-polyacrylamide (15%) gel electrophoresis of N- and C-fragments Mi-CK.

Left: Ultrasound/Benzonase® endonuclease – treatment of washed inclusion bodies (strongly overloaded gel; practically no contamination visible).

Right: Renatured fragments after cation chromatography for purification and homogenization. Neither the raw inclusion body fraction nor the end product shows signs of the presence of proteolytic degradation products.

Experimental design

Expression of the fragments took place over 5 hours at 37 °C. The *E.coli* cells were harvested by centrifugation, and washed with buffer P (PBS, pH 7.2 + 5 mM EDTA). The periplasmatic proteins were removed by swelling with distilled water on ice for 10 minutes followed by another centrifugation step. The cells were then lysed (on ice) with an ultrasonic probe in buffer containing only 1 U Benzonase® endonuclease per ml. The lysate was incubated for 30 minutes at 37 °C to digest the nucleic acids. Following centrifugation at 3,000 x g (15 minutes, 4 °C), the pellet, which contained the inclusion bodies as well as the *E.coli* cell fragments, was resuspended in buffer W (buffer + 25% sucrose + 1% Triton® X-100 + 1 U Benzonase® endonuclease per ml). It was then resubjected to ultrasonification and recentrifugation at 23,000 x g (10 minutes, 4 °C). The ultrasound/centrifugation procedure was then repeated twice. The inclusion bodies remain completely stable during this treatment. Excess detergent and Benzonase® endonuclease were then removed by washing twice with distilled water. The resulting inclusion body preparation proved to be very pure (see Fig. 12). The inclusion bodies were dissolved in 8 M urea and while still in a denatured state, subjected to one-step cation chromatography and purified to homogeneity.

Results

When renatured by dilution or dialysis (no protease inhibitors were used) no proteolysis occurred; the purified fragments were 100% intact (see Fig. 12) and resulted in enzymatically active Mi-CK when mixed in vitro. From a total of 1.6 L of bacterial culture, several hundred milligrams of both purified fragments could be obtained within two days (including both expression and purification). No proteolytic degradation of Mi-CK fragments was observed during refolding and purification. That indicates the successful and complete removal of any adhering proteases from the inclusion bodies.

Example 5: Use of Benzonase® endonuclease for sample preparation in two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis is a powerful technique for the high-resolution separation of complex mixtures of proteins. Nucleic acids are negatively charged molecules. They tend to form complexes by electrostatic interactions with positively charged domains on the surface of proteins. The formation and shape of these adducts usually cannot be predicted. These nucleic acid-protein complexes migrate differently in an electric field when compared to the pure protein. In addition to other effects this may lead to band shifts in the expected protein pattern and cause poor resolution in 2-D gel electrophoresis. Sample pretreatment with Benzonase® endonuclease (50 U per 100 µl of cell lysate) strongly reduces horizontal streaking and significantly enhances the resolution of electrophoretic separation (Fig. 13). It is important to note that the presence of Benzonase® endonuclease could not be detected on the gel due to the low amount of enzyme required.

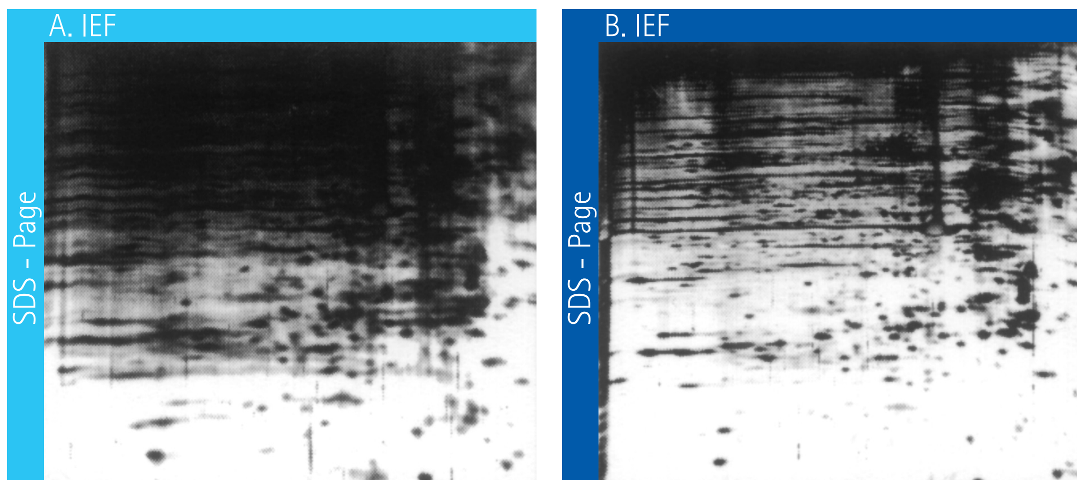


Figure 13.
Silver-stained two-dimensional gel electrophoretic separation of bacterial cells (Proteus vulgaris)

a) without use of Benzonase® endonuclease
b) by use of Benzonase® endonuclease

Note: The presence of Benzonase® endonuclease could not be detected on the gel.

Example 6: Prevention of cell clumping

A newer application of Benzonase® endonuclease is its incorporation into cell culture media to prevent cell clumping, especially when thawing frozen cell samples. Not only is Benzonase® endonuclease free of protease activity, but it poses no threat to healthy cells making it ideal for such a role.

Recent studies demonstrate utility of Benzonase® endonuclease in developing vaccines against some of the world's most serious infectious diseases. Peripheral blood mononuclear cells (PBMCs) isolated from whole blood have important applications in vaccine research, e.g., during quantification of vaccine-induced T-cell responses. T-cell response assay methods initially require freshly isolated cells for optimal signal detection, posing a serious practical limitation for sample handling during large clinical trials. However, frozen PBMCs (particularly PBMCs prepared from stored blood) tend to clump together upon thawing, preventing further analysis. In 2001, Smith et al. (1) demonstrated that inclusion of Benzonase® endonuclease in PBMC thawing buffer prevented cell clumping, allowing implementation of PBMC cryopreservation. This method has since been widely adopted in vaccine evaluation studies (2, 3).

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2. Huaman, M.C., et al. (2008). *J. Immunol.* **180**, 1451.
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5 Frequently Asked Questions

Working with Benzonase® endonuclease for the first time – or using it in a new application? Consider this section your first stop when looking for answers. If you don't find what you're looking for here, please get in touch with your local Merck Millipore contact or alternatively see our website www.merckmillipore.com/benzonase.

Which quality/quantity of Benzonase® endonuclease will be adequate for a certain application?

There are several parameters which influence the activity of Benzonase® endonuclease (see Fig. 14). Hence, the optimum conditions will vary from process to process and need to be determined experimentally. For viscosity reduction, Benzonase® endonuclease, purity grade II (≥90%) will often be sufficient.

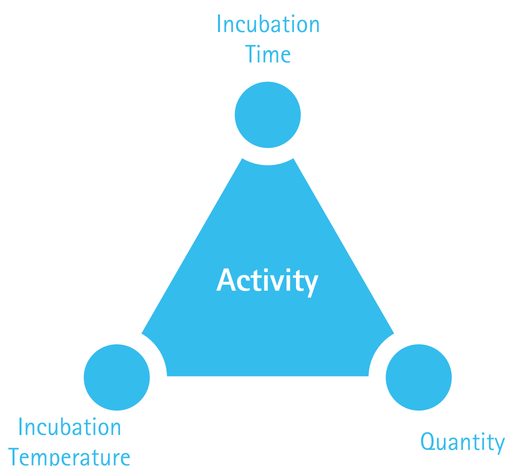

Figure 14. Benzonase® endonuclease triangle

At which step do I have to introduce Benzonase® endonuclease in my process?

The answer to this question will vary depending on why you are using Benzonase® endonuclease. The example applications given will hopefully help you answer this question. However, as a general rule, Benzonase® endonuclease is usually best added after the fermentation step and before the capture step.

How much more Benzonase® endonuclease do I have to add if I am working at low temperatures?

At temperatures below 37 °C the efficiency of Benzonase® endonuclease decreases, as illustrated in the section on the temperature stability of Benzonase® endonuclease (see pages 6–7). The amount needed to compensate for this decrease in efficiency will vary from process to process and on the other parameters present. Often, increasing another parameter, such as incubation time, can compensate without needing to increase the quantity of Benzonase® endonuclease used.

Why is Benzonase® endonuclease not working? What will inhibit its activity?

Benzonase® endonuclease is active under a wide range of operating conditions (see page 8, Table 1), however a concentration of 1–2 mM Mg^{2+} is essential for the activity of Benzonase® endonuclease.

Mn^{2+} can substitute Mg^{2+}, however the enzyme will only reach its optimum activity in the presence of Mg^{2+}. It is inhibited (approximately 50% activity) by monovalent cation concentrations >300 mM, phosphate concentrations >100 mM, and by ammonium sulfate concentrations >100 mM. In addition, concentrations of >1 mM EDTA will also inhibit Benzonase® endonuclease activity.

I observe a loss of activity – why?

Benzonase® endonuclease is usually very stable, however in rare cases a loss of activity can be observed. There are several possible reasons for this; irreversible inactivation can be due to the presence of denaturing agents in the sample, e.g. proteases, or alternatively due to incorrect storage. Reversible inactivation is commonly due to the presence of chelating agents such as EDTA, which remove essential magnesium ions.

My Benzonase® endonuclease was left out on the bench all weekend. Is it still good?

We have done extensive stability testing on Benzonase® endonuclease, and find that it is extremely stable. Even with extended incubations at 37 °C, Benzonase® endonuclease maintained >90% of activity for several weeks. After a storage time of 11 weeks at 25 °C (60% RH) the activity even remained unchanged (see page 7, Fig. 2b).

How do I inhibit Benzonase® endonuclease activity?

There are process additives/agents that affect Benzonase® endonuclease activity – for example it can be inhibited by high salts, like >300 mM monovalent cations, >100 mM phosphate, >100 mM ammonium sulfate, >100 mM guanidine HCl. Other known inhibitors are chelating agents, like EDTA, which could cause loss of free Mg^{2+}-ions (EDTA concentrations >1 mM have shown to inhibit the enzymatic reaction). This can be reversed by adding more $MgCl_2$.

How do I remove Benzonase® endonuclease?

Removal of Benzonase® endonuclease can be accomplished by several downstream units of operation like depth filtration for clarification, tangential flow filtration (TFF) for concentration & diafiltration and chromatography (IEX, SEC, HIC). Please see Appendix, Chapter 2 "Removal of Benzonase® endonuclease" (page 22) for further information.

Is Benzonase® endonuclease safe?

Yes, toxicological studies with Benzonase® endonuclease have been performed (internal reports available). The systemic toxicity after single application was investigated in mice and rats: no toxic effects have been observed even at very high doses. In addition no mutagenic potential has been observed in mice treated intravenously even with a very high dose of Benzonase® endonuclease.

Is Benzonase® endonuclease free of protease activity?

Yes, Benzonase® endonuclease is supplied without detectable protease activity and is hence not degraded during its "work." The presence of protease in the sample itself will, however, result in irreversible degradation of the Benzonase® endonuclease.

Is Benzonase® endonuclease compatible with protease inhibitor cocktails?

Yes. However, caution should be exercised since many protease inhibitor cocktails include EDTA. Concentrations of greater than 1 mM EDTA will inhibit the activity of Benzonase® endonuclease.

In which step should I introduce Benzonase® endonuclease to my process?

This varies depending on the application. However, as a general rule, Benzonase® endonuclease is usually best added after the fermentation step and before the capture step.

Do you offer immobilized Benzonase® endonuclease?

No. All efforts to bind Benzonase® endonuclease to a support that meets the demands of a commercial product with respect to activity, stability, and regulatory requirements have so far been unsuccessful.

Why is the filling range volume of the 5-million-unit tubes not specified?

As the specific activity (U/ml) of Benzonase® endonuclease may vary between production lots, we decided to specify the units per tube but not the volume. The volume per tube can be easily calculated from the specific activity information on the Certificate of Analysis (CoA).

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7 Ordering Information

Benzonase® endonuclease is manufactured in Denmark. It is distributed worldwide exclusively by Merck KGaA, its affiliates, and selected dealers. Benzonase® endonuclease is supplied in 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 20 mM NaCl, and 50% (v/v) glycerol in three different package sizes and two purity grades.

Designation	Package size	Ord. No.
Benzonase® endonuclease, purity grade II (≥90%), for biotechnology	100,000 U/vial	1.01654.0001
Benzonase® endonuclease, purity grade II (≥90%), for biotechnology	500,000 U/vial	1.01656.0001
Benzonase® endonuclease, purity grade I (≥99%) suitable for biopharmaceutical production EMPROVE® bio	100,000 U/vial	1.01695.0001
Benzonase® endonuclease, purity grade I (≥99%) suitable for biopharmaceutical production EMPROVE® bio	500,000 U/vial	1.01697.0001
Benzonase® endonuclease, purity grade I (≥99%) suitable for biopharmaceutical production EMPROVE® bio	5,000,000 U/vial	1.01697.0010
Benzonase® endonuclease ELISA Kit II for the immunological detection of Benzonase® endonuclease	5 plates (8 x 12) plus reagents	1.01681.0001

Appendix

8

1 Benzonase® endonuclease Standard Activity Assay (volume activity)

Calculation principle

Benzonase® endonuclease degrades sonicated DNA into oligonucleotides three to five base pairs in length. The production of these oligonucleotides leads to an increase in absorbance at 260 nm.

Unit definition

One unit corresponds to the amount of enzyme required to produce a change in absorbance at 260 nm of 1.0 in the time of 30 minutes, under optimum conditions with excess substrate (see below for details).

Reagents and solutions

1. Reagent A (1 mM $MgCl_2$, 0.1 mg/ml BSA, in 50 mM Tris, pH 8.0)

Dissolve 3.0 g Tris (Cat. No. 648310) in about 480 ml of redistilled water, adjust to pH 8.0 with 1.0 mol/l hydrochloric acid (Cat. No. 1.09057), and make up to 500 ml with redistilled water (results in 0.05 mol/l Tris/HCl buffer solution). Subsequently add 0.10 g of magnesium chloride hexahydrate (Cat. No. 1.05833) and 50 mg of albumin fraction V (Cat. No. 1.12018).

2. Reagent B (1 µg/ml DNA)

Dissolve herring sperm DNA (Cat. No. D1815, Promega) in reagent A to a final concentration of 1 mg/ml.

3. Perchloric acid solution (4%)

Dilute 5.63 ml 70 – 72% perchloric acid (Cat. No. 1.00519) with redistilled water to 100.0 ml.

4. Enzyme (Benzonase® endonuclease) solution

Dilute 16 µl of the sample with ice-cold reagent A to 100.0 ml and make up 10.0 ml of this solution with reagent A to 50.0 ml.

Table 6.

Assay procedure

Pipette into test tubes
We recommend that each test series should be performed in duplicate along with a blank value.

	Sample	Blank value
Reagent B	2.500 ml	2.500 ml
Enzyme solution	0.125 ml	–
Reagent A	–	0.125 ml
Incubate in a water bath at 37 °C		
After 15 min, 30 min, 45 min and 60 min, pipette 0.500 ml each of the incubation preparation into an Eppendorf tube (1.4 ml) containing 0.500 ml of perchloric acid (3). Mix, incubate on ice 30 to 60 min, and centrifuge at 14,000 rpm, for 6 min at 4 °C. Transfer the supernatant into new Eppendorf tubes and measure the absorbance of the samples against the blank.		
Wave length: 260 nm	Optical path length: 1 cm	

Calculation

$$U/\mu l = \frac{\Delta A \cdot 30 \cdot V \cdot 2 \cdot F}{t \cdot v \cdot 1000}$$

$$\rightarrow U/\mu l = \frac{\Delta A \cdot 30 \cdot 2.625 \cdot 2 \cdot 31250}{t \cdot v \cdot 1000}$$

$$\rightarrow U/\mu l = \frac{\Delta A \cdot 39375}{t}$$

where

A = absorbance of the measuring solution at time t
30 = one unit is defined at 30 min
V = total volume of the incubation mixture
2 = dilution factor of the measuring solution
F = dilution factor sample solution
t = incubation time of the measuring solution
v = sample volume
1000 = conversion factor of ml to µl

2 Removal of Benzonase® endonuclease

Removal of Benzonase® endonuclease from the manufacturing process can be accomplished in several ways during downstream purification operations, such as tangential flow filtration (TFF) or chromatography. Successful removal of Benzonase® endonuclease can be shown by demonstrating no residual nuclease activity (does not detect residual non-active Benzonase® endonuclease) and through ELISA for detection of the total residual endonuclease (active and non-active). Drug product manufacturers using Benzonase® endonuclease in the manufacturing process of their drug need to demonstrate data related to its safety/toxicity and measure residual endonuclease that may be present in the final preparation.

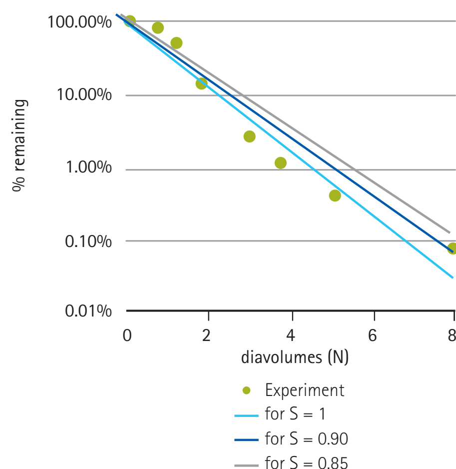


Figure 15.
Benzonase® endonuclease TFF diafiltration

TFF diafiltration with the example of a viral vaccine production process: up to >99.5% clearance of Benzonase® endonuclease at 5 diavolumes and >99.9% after 8 diavolumes using a 300 kD membrane. The overall diafiltration profile follows close to a theoretical sieving value of 1.

Benzonase® endonuclease is easily removed with the filtrate while the drug product can be retained by using TFF and by selection of appropriate membrane cut-offs. Benzonase® endonuclease can be removed from the process by diafiltration to concentrations below detectable limit (Fig. 15).

Depending on purification challenges, ion-exchange chromatography may be used alternatively or additionally to TFF for removal of Benzonase® endonuclease. Ion chromatography is well known for removal of residual endonuclease from drug products. With a pI of 6.85, it will typically flow through anion exchange chromatography (AEX) while the drug product may be bound to the AEX column or in case it is bound as well, elutes separately. Several anion exchange resins using a variety of sample and equilibration buffers are illustrated for Benzonase® endonuclease removal in Table 7.

Cation exchange chromatography (CEX) can also be effective in removal of Benzonase® endonuclease but the operating range might be smaller. Table 8 provides a reference list for a few chromatography media and conditions that are suitable for the removal of Benzonase® endonuclease.

Table 7.
Anion exchange chromatography for removal of Benzonase® endonuclease

Fractogel® EMD resin	pH	Sample & equilibration buffer	Benzonase® endonuclease	BSA
TMAE*	7.0	50 mM Tris / 200 mM NaCl	not bound	not bound
TMAE	7.0	50 mM Tris / 50 mM NaCl	not bound	bound
TMAE	8.0	50 mM Tris / 250 mM NaCl	not bound	not bound
TMAE	8.0	50 mM Tris / 100 mM NaCl	not bound	bound
TMAE	9.0	50 mM Tris / 200 mM NaCl	not bound	partially bound
TMAE	9.0	50 mM Tris / 100 mM NaCl	not bound	bound
DEAE*	7.0	50 mM Tris / 200 mM NaCl	not bound	not bound
DEAE	7.0	50 mM Tris / 50 mM NaCl	not bound	bound
DEAE	8.0	50 mM Tris / 250 mM NaCl	not bound	not bound
DEAE	8.0	50 mM Tris / 100 mM NaCl	not bound	bound
DEAE	9.0	50 mM Tris / 250 mM NaCl	not bound	not bound
DEAE	9.0	50 mM Tris / 50 mM NaCl	not bound	bound
DMAE*	8.0	50 mM Tris / 250 mM NaCl	not bound	partially bound
DMAE	8.0	50 mM Tris / 50 mM NaCl	not bound	bound

* TMAE = Trimethylammoniumethyl; DEAE = Diethylaminoethyl; DMAE = Dimethylaminoethyl

Fractogel® EMD resin	pH	Sample and equilibration buffer	Benzonase® endonuclease
SO_3^-	6.0	20 mM phosphate / 100 mM NaCl	bound
SO_3^-	6.0	20 mM phosphate / 200 mM NaCl	not bound
SO_3^-	5.0	20 mM acetate / 200 mM NaCl	bound
SO_3^-	5.0	20 mM acetate / 700 mM NaCl	not bound
SO_3^-	4.0	20 mM acetate / 300 mM NaCl	bound
SO_3^-	4.0	20 mM acetate / 800 mM NaCl	not bound
COO^-	6.0	20 mM phosphate / 0 mM NaCl	not bound
COO^-	5.0	20 mM acetate / 40 mM NaCl	bound
COO^-	5.0	20 mM acetate / 100 mM NaCl	not bound
COO^-	4.0	20 mM acetate / 150 mM NaCl	partially bound
COO^-	4.0	20 mM acetate/ 400 mM NaCl	not bound

Table 8.
Cation exchange chromatography for removal of Benzonase® endonuclease

Gousseinov, E., et al. (2013). Nucleic Acid Impurity Reduction in Viral Vaccine Manufacture. *Bioprocess Int.* in press.

Eglon, M., et al. (2009). Purification of adenoviral vectors by combined anion exchange and gel filtration chromatography. *J. Gene Med.* **11**, 978.

3 Detection of Benzonase® endonuclease – Benzonase® ELISA Kit II

This assay detects and quantifies the amount of Benzonase® endonuclease present in samples, thus allowing proof of its removal. The use of specific antibodies against Benzonase® endonuclease results in a precise assay with a sensitivity of 0.2 ng/ml. This corresponds to less than 1 ppm in the presence of other proteins (at concentrations higher than 0.5 mg/ml). Cross-reactions with *E.coli, Pichia pastoris*, normal mouse serum, bovine serum albumin, and fetal calf serum are less than 1%.

- Fast and simple to use
- Shelf life of 12 months
- Low detection limit (0.2 ng/ml)
- Single strip plates (8 x 12)

How does the assay work?

The assay consists of polystyrene microtiter plates coated with polyclonal antibodies, which "capture" Benzonase® endonuclease present in the sample. Horseradish peroxidase conjugated antibodies are then added. The resulting complex is detected by the formation of a visible, yellow product following the addition of TMB (3, 3′, 5, 5′-tetramethylbenzidine). The reaction is stopped by the addition of 0.2 M H_2SO_4 and the result can be read visually or using an ELISA plate reader at 450 nm.

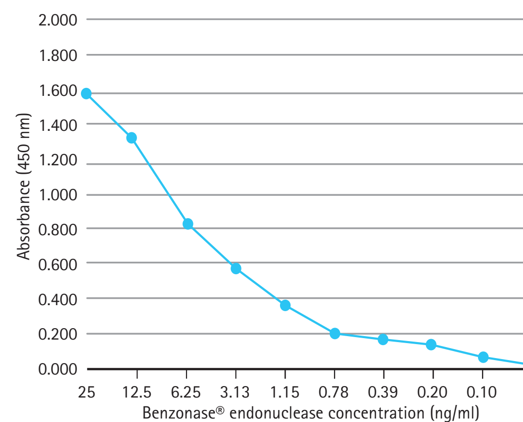
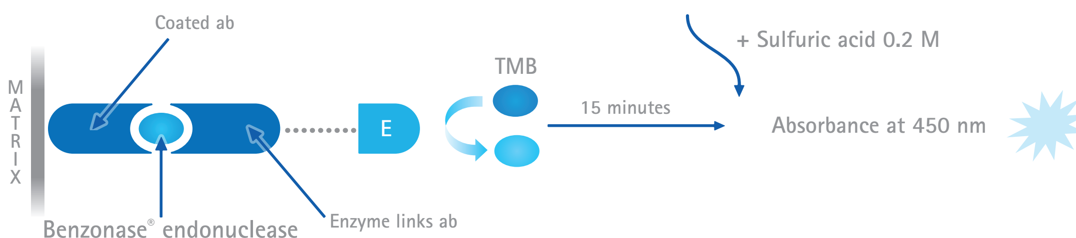


Figure 16.
Example of a typical calibration curve for Benzonase® endonuclease, purity grade I (≥99%) in the range of 0.11 to 25 ng/ml Benzonase® endonuclease concentration absorbance (450 nm).



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