

Benzonase[®] endonuclease

The Smart Solution for DNA Removal

Advancing Your Life Sciences –
From Discovery to Launch[™]



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endonuclease

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How to meet the highest possible purity standards for biopharmaceuticals?

How to increase downstream processing yields?

Benzonase[®] endonuclease



There is only one effective biochemical method to remove DNA and RNA, both in the laboratory and in industrial scale processes. It is called Benzonase[®] endonuclease.

Benzonase[®] endonuclease is a unique, genetically engineered endonuclease offering a variety of advantages over existing methods of nucleic acid removal.

Benzonase[®] endonuclease is manufactured and exclusively distributed by Merck KGaA Darmstadt, Germany and its associates.

Benzonase[®] endonuclease has proven its value in the laboratory for over ten years and is currently successfully applied in various processes throughout the pharmaceutical and biotechnological industry.





Benzonase® endonuclease

Benzonase® endonuclease

- attacks and degrades all forms of DNA and RNA.
- is free of detectable proteolytic activity.
- is effective over a wide range of operating conditions.
- possesses an exceptionally high specific activity.
- is manufactured under strictly regulated conditions in order to meet industrial requirements for a reliable supply and consistent high quality.
- is ideal for a variety of applications including:
 - purification of proteins and other biologicals.
 - reduction of viscosity caused by nucleic acids.
 - sample preparation in electrophoresis and chromatography

At a glance

Benzonase® endonuclease is a genetically engineered endonuclease produced in *Escherichia coli* strain W3110, a mutant of strain K12, which contains the proprietary pNUC1 production plasmid [1, 2]. This plasmid encodes an endonuclease normally expressed in *Serratia marcescens*. The production techniques used to manufacture Benzonase® endonuclease ensure a product of exceptionally high purity and activity. These techniques enable the enzyme to be supplied without measurable protease activity and without 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 two different grades of purity

**Benzonase® endonuclease
Purity grade I (Ultra pure grade)**

- more than 99% pure.

**Benzonase® endonuclease
Purity grade II (Pure grade)**

- more than 90% pure.

Regulatory information

US FDA regulations are regarded as the world's most demanding standards of quality and purity for biopharmaceuticals. There are no explicit FDA regulations governing the production and use of excipients such as Benzonase® endonuclease. However, at Merck KGaA Darmstadt, Germany we place a high value on the safety of our products. For this reason Benzonase® endonuclease is produced under cGMP conditions (we are currently in the process of obtaining full cGMP validation*). There also exists a DMF type II file for Benzonase® endonuclease at the US FDA (Reg. No. BMMF 5403). In addition to this Merck KGaA Darmstadt, Germany operates a quality management system according to DIN ISO 9001 and 14001.

Auxiliary materials

The finished product does not contain additives of animal origin such as stabilisers like bovine serum, albumin or gelatine. Benzonase® endonuclease is supplied in 50 % glycerol solution to prevent freezing of the preparation. The glycerol is of synthetic origin.

During production of Benzonase® endonuclease the fermentation medium contains casamino acids from bovine milk. This milk originates from countries with no recorded BSE cases in locally bred animals since 1990 and is considered fit for human consumption. We can supply on demand full batch documentation. This documentation fulfils current US FDA requirements*.

Viral safety

We use a well defined bacterial expression System (*Escherichia coli* strain W3110 a K12 mutant) for the preparation of Benzonase® endonuclease. The risk of viral contamination is regarded as negligible.

Microbiological safety

Each batch, with the exception of packages containing 10,000 units, is tested for the absence of aerobic bacteria, yeasts and moulds according to a modified EP method.

Endotoxin testing

Each batch of Benzonase® endonuclease purity grade I is tested for endotoxins by an independent, accredited laboratory using the well known LAL-test. The total endotoxin level is below 0.25 EU per 1.000 units.

This field of regulatory information is constantly changing, for the latest updates please contact us at processing@merck.de

Product Description



Product quality assurance: Using Benzonase® endonuclease to remove DNA/RNA

FDA guidelines for the manufacture of recombinant biologicals for therapeutic use demand that nucleic acid contamination should be limited to 10 pg per dose (in the end product) [3, 4]. However, this depends completely on the pharmaceutical indication area of the drug. Generally, nucleic acid contamination is measured by using appropriate DNA or RNA probes to detect hybridizable nucleic acids or by other sensitive DNA assays.

Benzonase® endonuclease, when used under appropriate reaction conditions, will degrade all nucleic acid sequences down to oligonucleotides of approximately 3 to 5 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 results in a number of benefits including:

- reduced processing time

- increased yield of protein products
- improved separation of pellet and supernatant in centrifugations
- facilitated filtration of solutions, especially ultrafiltration
- increased efficiency of chromatographic purification steps (e.g. in expanded bed adsorption) [5].

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 [6–8]. 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 the yield.

Bioanalytical applications: Using Benzonase® endonuclease for sample preparation

Treating samples containing nucleic acids with Benzonase® endonuclease before they are used for analysis, e.g. ELISA, chromatography or two-dimensional electrophoresis (protein mapping) and footprint analysis [9] provides a number of benefits including:

- improved resolution, due to reduced interference from charged nucleic acid fragments present in the sample,
- increased recovery, due to reduced entrapment of product after reduction of sample viscosity.

These benefits are especially important when working with small sample volumes such as Mini-Gel electrophoresis or HPLC separations.

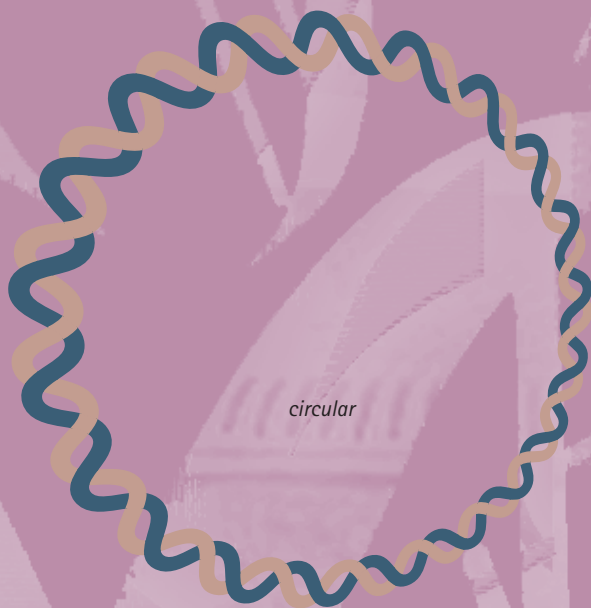
Benzonase[®] endonuclease



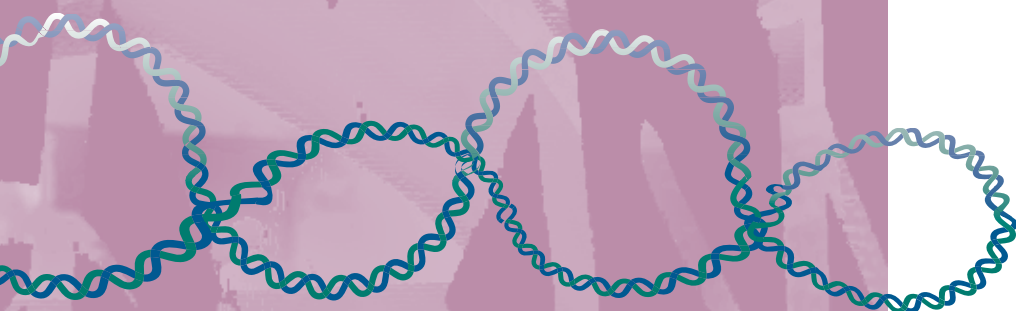
single strand



double strand



circular



circular supercoiled

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. Mg²⁺ (1-2 mM) is required for enzyme 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 3 to 8 bases in length.

Activity

Specific activity for Benzonase[®] endonuclease is measured under standard assay conditions (see below). Minimum specific activity for Benzonase[®] endonuclease purity grade II (90 %) is 1.0×10^6 units/mg protein. Minimum specific activity for Benzonase[®] endonuclease purity grade I (99 %) is 1.1×10^6 units/mg protein.

Enzyme Characteristics

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. 2.

We do not recommend repeated freeze/thaw cycles and storage at temperatures lower than -20°C, data can be supplied that shows no loss of activity during shipment.

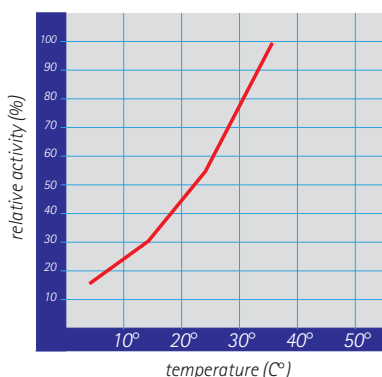
Product purity

Analysis of Benzonase® endonuclease Pure Grade (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 Ultra Pure Grade (purity grade I, 99 %) is produced by chromatographic purification of Benzonase® endonuclease Pure Grade. Analysis of Benzonase® endonuclease Ultra Pure Grade 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 1: Effect of temperature on Benzonase® endonuclease activity*



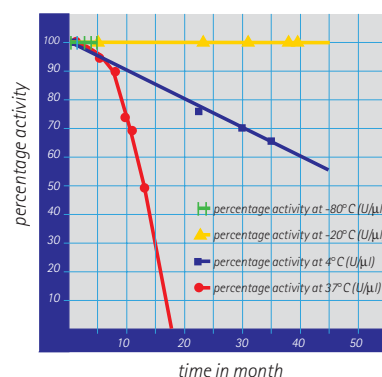
*Standard assay conditions
(Substrate is DNA)

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

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



30 minutes under standard assay conditions.

For a detailed description of the standard assay and additional sensitive nuclease assays see Appendix I.

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 activities is monitored by a highly sensitive and validated assay using a resorufin-labelled casein (Cat. No. 1.24852) as substrate (detailed instructions are available on request).

Benzonase[®] endonuclease

Operating conditions

Benzonase[®] endonuclease retains its activity in a wide range of operating conditions, as specified in the following table and figures 3 to 6.

Table 1: Operating conditions for Benzonase[®] endonuclease

Condition	Optimal*	Effective**
Mg ²⁺ concentration	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
β-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 operating range in which Benzonase[®] endonuclease retains ≥ 90 % of its activity.
 ** "Effective" is defined as the operating range in which Benzonase[®] endonuclease retains > 15 % of its activity.

Figure 3: Effect of magnesium and manganese ion concentrations on Benzonase[®] endonuclease activity*

A concentration of 1 to 2 mM Mg²⁺ or Mn²⁺ is essential for the activity of Benzonase[®] endonuclease. Mg²⁺ is preferred because it enables the enzyme to reach its optimal level of activity. Ca²⁺ and Sr²⁺ do not effect the activity of the enzyme.

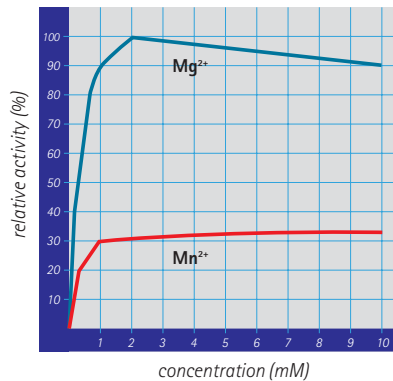


Figure 5: Effect of monovalent cations on Benzonase[®] endonuclease activity*

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

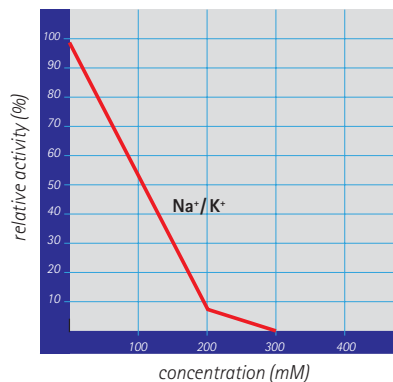


Figure 4: Effect of pH on Benzonase[®] endonuclease activity*

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.

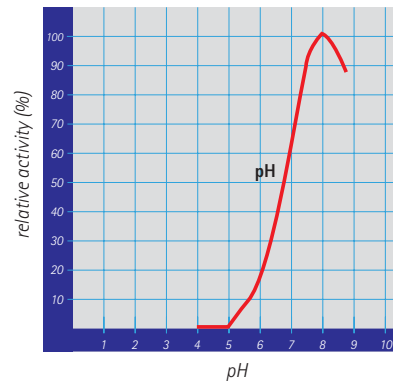
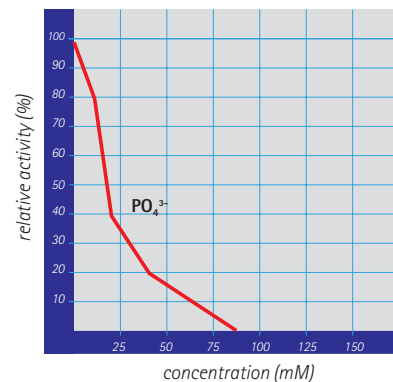


Figure 6: Effect of phosphate ion concentration on Benzonase[®] endonuclease activity*

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 Mg²⁺ ions. PMSF in a concentration of 1 mM does not inhibit Benzonase[®] endonuclease.

* Standard assay conditions = 100 % activity. Substrate is DNA.

** Effects were determined by measuring the change in absorbance that occurs when oligonucleotides are released into solution.

Enzyme Characteristics

Benzonase® endonuclease is active in the presence of ionic and non-ionic detergents, reducing agents, and urea. This is illustrated in Figures 7 to 10.

* Standard assay conditions = 100 % activity. Substrate is DNA.

** Effects were determined by measuring the change in absorbance that occurs when oligonucleotides are released into solution.

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.

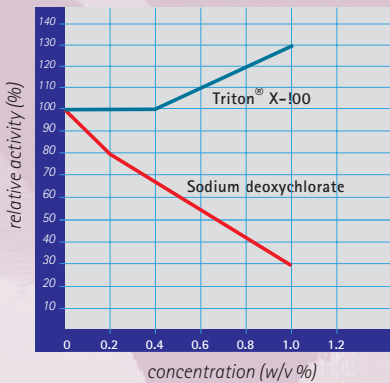


Figure 8a and 8b: Effect of different SDS concentrations on Benzonase® endonuclease activity

Benzonase® endonuclease retains 100 % of its activity in 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. Relevant Benzonase® endonuclease amounts are given in units/ml.

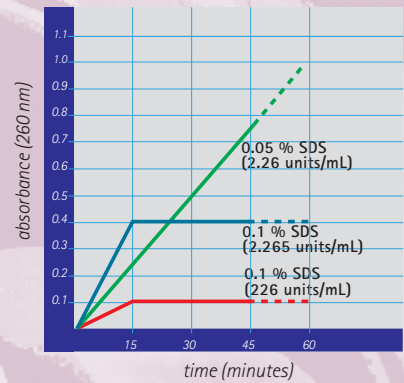
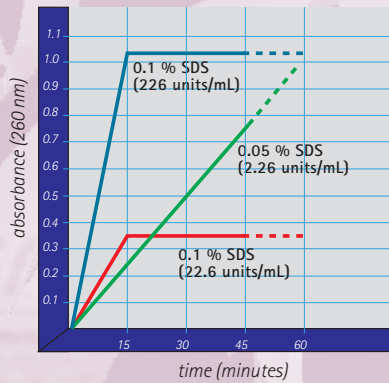


Figure 9a and 9b: Effect of different concentrations of urea on Benzonase® endonuclease activity*

Benzonase® endonuclease is activated by urea at concentrations up to approximately 6M. At 6M urea, enzyme activity first increases, then decreases over time. At 7M urea, Benzonase® endonuclease denatures after 15 minutes, and activity is lost. However, significant degradation of nucleic acids occurs before the enzyme is inactivated. Higher concentrations of Benzonase® endonuclease can partially compensate the effects of 7M urea. Benzonase® endonuclease concentrations are given in units/ml.

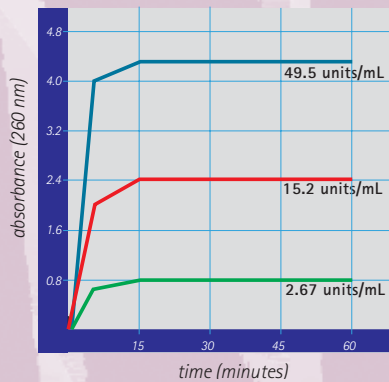
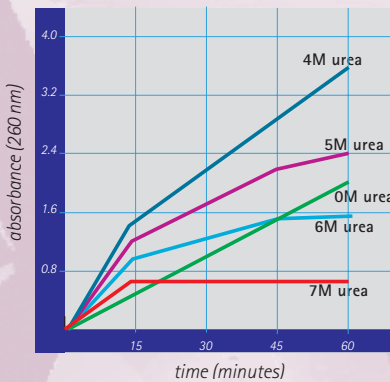
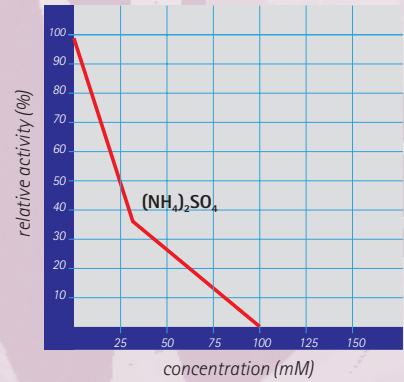


Figure 10: Effect of ammonium sulfate on Benzonase® endonuclease activity**

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





Benzonase[®] endonuclease

The following section contains examples of typical applications of Benzonase[®] endonuclease. It is intended to be used as a guide rather than as detailed method instructions. It is recommended that the optimum conditions for each process be experimentally determined on a small scale first.

"Traditional" methods for the removal of nucleic acids such as extraction or precipitation are not specific. Denaturation and precipitation results in reduced protein yields.

Previous solutions to this problem were the use of DNase or RNase, however this approach can no longer be recommended.

- neither DNase nor RNase possess a specific activity as high as that of Benzonase[®] endonuclease.
- neither DNase nor RNase are available in comparable purity grades
- Benzonase[®] endonuclease is the only known endonuclease which degrades all types of nucleic acids
- Benzonase[®] endonuclease has been genetically engineered and is expressed *E.coli*. It is free of detectable proteolytic activities.
- there is no other endonuclease produced in bulk quantities for use on a technical scale
- Benzonase[®] endonuclease has been especially designed for its application in biotechnological processing and biopharmaceutical production.

Example 1: Elimination of nucleic acids from recombinant proteins

Most recombinant products, but especially those intended for therapeutic use, 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 to 5 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 MgCl₂, 0.1 mg/ml BSA, pH 8.0). The progress of the reaction was assayed as follows: At different time intervals (0-30 hrs) aliquots of 10 µl (initially containing 500 ng of DNA) were applied to a nitrocellulose membrane and hybri-

dized with a ³²P-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 semiquantitative evaluation of the residual hybridizable DNA.

Typical Applications

Figure 11: illustrates the results of the experiment by showing the autoradiograms for the degradation experiments described above.



Results

1 Effect of Benzonase® endonuclease concentration

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 4 hours at a concentration of 90 U/ml, 99.95 % of the DNA can no longer be hybridised. However reducing the initial enzyme concentration by 90 %, i.e. to 9 U/ml, still yields 99 % of the DNA as non - hybridisable.

1 Residual hybridizable DNA (in pg) after incubation for Benzonase® endonuclease concentration

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 (<10pg)

2 Effect of temperature

Decreasing the incubation temperature results in increased incubation times.

2 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)

3 Effect of inhibition

Since PBS is commonly used in bio-processes, the activity of Benzonase® endonuclease in PBS compared to that in Tris was determined.

3 Residual hybridizable DNA (in pg) after incubation for

	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 U/ml · Temperature: 23 °C

The experiment shows that complete DNA fragment elimination can be achieved using Benzonase® endonuclease. Due to its high specific activity ($\geq 1 \times 10^6$ 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 optimisation of the conditions should be based on reaction time,

temperature and concentration. Note that the three factors influence each other (see page 20 Benzonase® endonuclease triangle).

Example 2: Viscosity reduction I

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 minimize target protein loss. Benzonase[®] endonuclease achieves rapid and efficient liquefaction of viscous cell extracts.

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). This corresponds to a final concentration of 50 units/ml. All samples were incubated at 4°C for 5 min., and then centrifuged at 10,000 x g for 1 min. This experiment was repeated using two 1 ml aliquots.

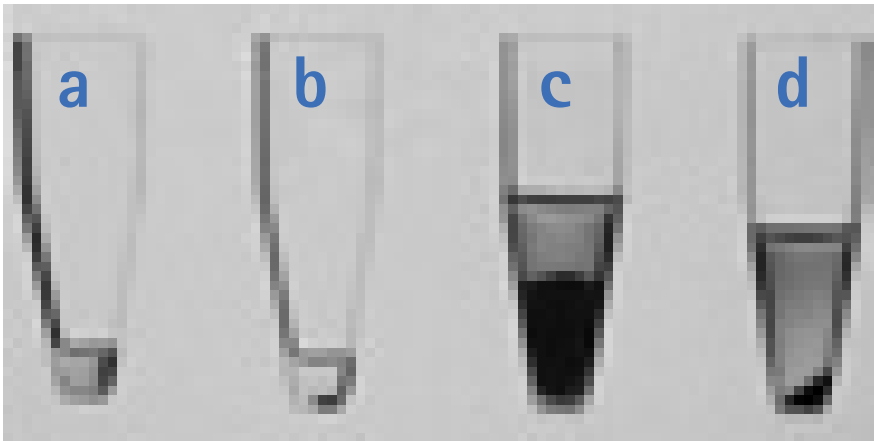


Figure 12:

Protein extracts of human granulocytes
a) without Benzonase[®] endonuclease,
b) with Benzonase[®] endonuclease (5 units);
c), d) same conditions as in a), b), but
volumes 10 times increased.

Results

After centrifugation the samples without Benzonase[®] endonuclease (a & c) retain a high viscosity, with no clear demarcation between supernatant and pellet. However, the samples containing Benzonase[®] endonuclease (b & 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.

Typical Applications

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.

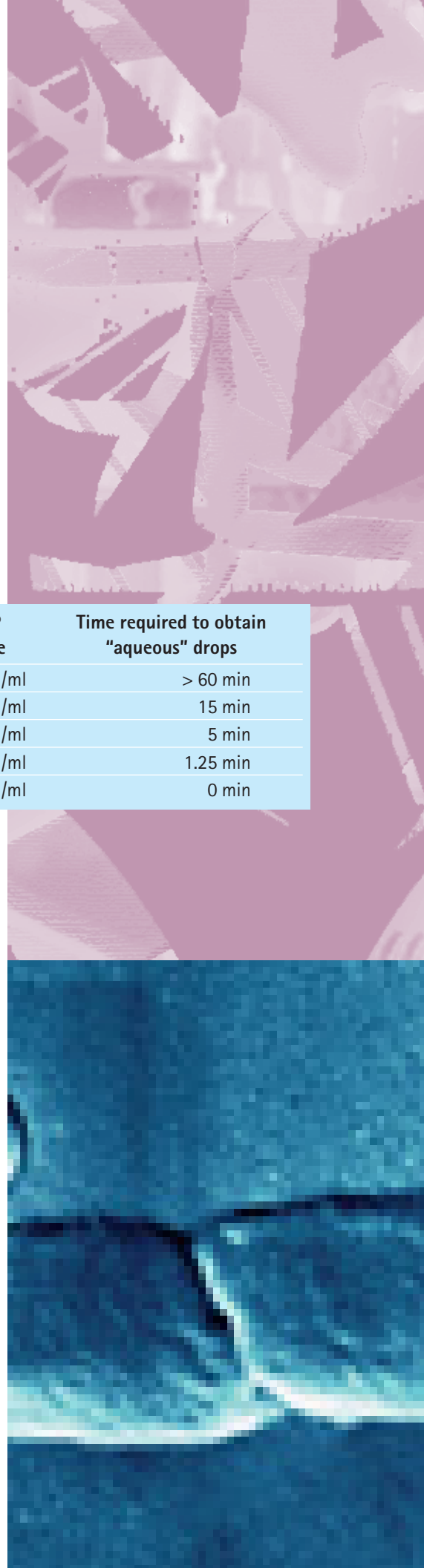
The aliquots were passed through a French press at 10,000 psi and immediately incubated at 0 °C. Changes in viscosity were visually followed by measuring viscoelastic properties with a pipette. The time of release from the press was taken as start of the reaction ($t = 0$).

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 like drops decreases.

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

Figure 13: *E.coli* cells



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. This 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 for the purpose of protein folding experiments. The appropriate cDNA fragments were incorporated into suitable expression plasmids and expressed in *E. coli*. Both fragments were then successfully produced in large quantities by the bacteria, however they were in the form of inclusion bodies. An additional problem is that the unlike the wild type MiCK, the soluble form of the fragments is highly sensitive to proteolytic activity [10].

Experimental design

Expression of the fragments took place over 5 h 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 min at 37 °C to digest the nucleic acids. Following centrifugation at 3,000 x g (15 min., 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/ml). It was then resubjected to ultrasonification and recentrifugation at 23,000 x g (10min, 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. 14). The inclusion bodies were dissolved in 8M 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. 14) 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).

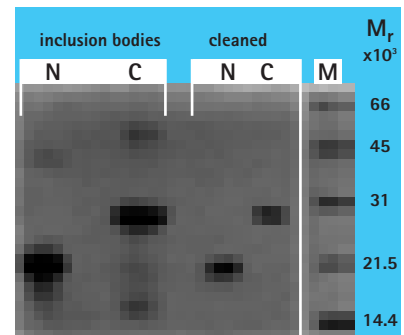


Figure 14: SDS-polyacrylamide (15 %) gel electrophoresis of N- and C-fragments Mi-CK.

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.

Typical Applications

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

Two-dimensional gel electrophoresis is a powerful technique (e.g. in proteomics) 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 2D gel electrophoresis. Without going into the experimental

details (ref. [11]), the benefit of sample pretreatment with Benzonase® endonuclease can be demonstrated in brief: Strong horizontal streaking can be seen in Figure 15a. This is due to polypeptides, which are continuously breaking away from the nucleic acid-protein complexes and migrating through the gel. As illustrated in Figure 15b, the resolution of electrophoretic separation can be significantly improved by using Benzonase® endonuclease.

Benzonase® endonuclease was added to the lysis buffer (9.8 M urea, 4 % Triton® X-100, 100 mM DTT, 5 % ampholytes). 50 U per 100 µl of cell lysate were sufficient under these conditions. It is important to note that the presence of Benzonase® endonuclease could not be detected on the gel.

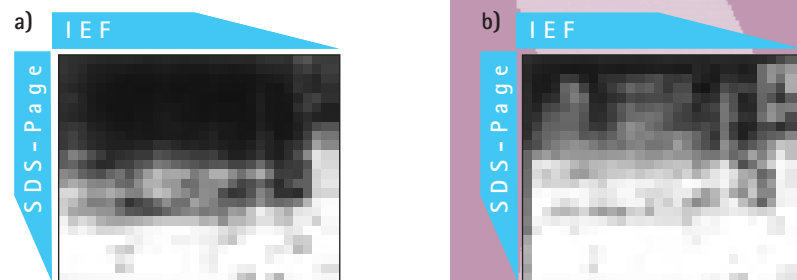


Figure 15: Silver-stained two dimensional gel electrophoretic separation of bacterial cells (*Proteus vulgaris*)
a) without use of Benzonase® endonuclease,
b) by use of Benzonase® endonuclease

Benzonase[®] endonuclease User references

Reference 1: Use of Benzonase[®] endonuclease in monoclonal antibody production

MedImmune, Inc. (USA) produces Synagis[®] (Palivizumab), a humanised monoclonal antibody (MAb) used to prevent serious lower respiratory tract diseases caused by respiratory syncytial virus (RSV) in high risk paediatric patients. During downstream processing of this API, MedImmune uses Benzonase[®] endonuclease to remove DNA contaminations.

For more details see ref. [13] or www.medimmune.com

Reference 2: Use of Benzonase[®] endonuclease in the downstream processing of recombinant FDH from *E.coli*

The Institute of Molecular Enzyme Technology at the Forschungszentrum Jülich, Heinrich-Heine University (Düsseldorf, Germany) has been developing a novel process for the production of Formate Dehydrogenase (FDH). The recovery process for FDH uses chromatography in conjunction with Benzonase[®] endonuclease. As a result, the yield of pure, industrial quality enzyme increases from 60 % to 85 %.

For more details see ref. [14, 15]

Reference 3: Use of Benzonase[®] endonuclease to reduce cell clumping

A novel 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.

For more details see ref [16]

Reference 4: Use of Benzonase[®] endonuclease in adenovirus purification

It has been known for a long time, that Benzonase[®] endonuclease can be used in adenovirus purification. Puresyn, Inc., uses Benzonase[®] endonuclease in their Adenopure[™] adenovirus purification kit for enhanced performance.

For further information please refer to their website www.puresyn.com.

For more details see ref. [18]



2020095 511

Product Name

Product Description

Product Description

Product Description

Product Description

This section is intended to answer the most frequently asked questions. For further information regarding any of these questions or other topics please contact us directly at processing@merck.de or alternatively see our website www.benzonase.de.

Benzonase® endonuclease

1) 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. 16 – Benzonase® endonuclease Triangle). Hence, the optimum conditions will vary from process to process and need to be determined experimentally. The example applications given, as well as the section concerning the operating conditions of Benzonase® endonuclease, will hopefully help you work out a strategy for process optimisation.

2) 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. The amount needed to compensate for this decrease in efficiency will vary from process to process and on the other parameters present (see Fig. 16 – Benzonase® endonuclease Triangle). Often, increasing another parameter, such as incubation time, can compensate without needing to increase the quantity of Benzonase® endonuclease used.

3) Do you offer immobilised 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.

Benzonase® endonuclease – Triangle

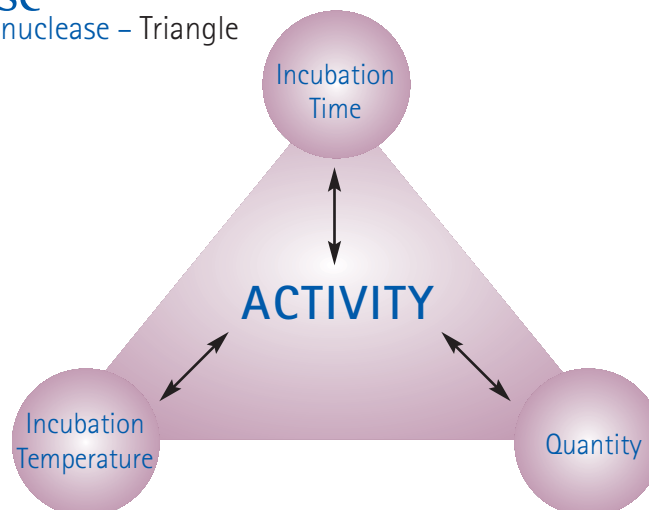


Figure 16:

Frequently Asked Questions

4) Why is Benzonase® endonuclease not working?

Benzonase® endonuclease is active under a wide range of operating conditions, however a concentration of 1-2 mM Mg²⁺ is essential for the activity of Benzonase® endonuclease. Therefore, the presence of a chelating agent such as EDTA will result in a lack of activity. Mn²⁺ can substitute for Mg²⁺, however the enzyme will only reach its optimum activity in the presence of Mg²⁺. For more details on the operating conditions of Benzonase® endonuclease (see pp 10).

5) 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, for example proteases (see point 6), or alternatively due to incorrect storage. Reversible inactivation is commonly due to the presence of a chelating agent such as EDTA, which remove essential magnesium ions (see also point 4).

6) 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 (see picture flow chart in Removal of Benzonase section).

7) 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 a protease in the sample itself will, however, result in irreversible degradation of the Benzonase® endonuclease.

8) How do I remove Benzonase® endonuclease?

Benzonase® endonuclease can easily be separated from your product using a variety of methods. Example strategies on how to remove Benzonase® endonuclease can be found in section "Removal of Benzonase® endonuclease" (see pages 28-30). The efficiency of removal of Benzonase® endonuclease can be determined using Benzonase® ELISA kit II (see Fig. 19, page 27).

9) How do I inhibit Benzonase® endonuclease activity?

So far there is no known inhibitor of Benzonase® endonuclease. Reversible inhibition can be achieved by using EDTA to chelate essential metal ions. Irreversible inactivation only occurs due to the presence of a protease in the sample or under extreme conditions (100mM NaOH at 70° for 30 minutes), which are also potentially harmful to the target product.

Benzonase[®] endonuclease

Ordering Information

Benzonase[®] endonuclease is manufactured in Denmark. It is distributed world-wide exclusively by Merck KGaA, its affiliates and selected dealers.

Benzonase[®] endonuclease is supplied in 20 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 20mM NaCl, and 50 % (v/v) glycerol in three different package sizes and two purity grades.

Cat. No.	Designation	Package size
1.01653.0001 1.01653.0002*	Benzonase [®] endonuclease purity grade II (≥ 90 %) ≥ 25 U/μl, for biochemistry	10,000 U/vial
1.01654.0001 1.01654.0002*	Benzonase [®] endonuclease purity grade II (≥ 90 %) ≥ 250 U/μl, for biotechnology	100,000 U/vial
1.01656.0001 1.01656.0002*	Benzonase [®] endonuclease purity grade II (≥ 90 %) ≥ 250 U/μl, for biotechnology	500,000 U/Vial
1.01694.0001 1.01694.0002*	Benzonase [®] endonuclease purity grade I (≥ 99 %) ≥ 25 U/μl, for biochemistry	10,000 U/vial
1.01695.0001 1.01695.0002*	Benzonase [®] endonuclease purity grade I (≥ 99 %) ≥ 250 U/μl, for biotechnology	100,000 U/vial
1.01697.0001 1.01697.0002*	Benzonase [®] endonuclease purity grade I (≥ 99 %) ≥ 250 U/μl, for biotechnology	500,000 U/Vial
1.01697.0010 1.01697.0012*	Benzonase [®] endonuclease purity grade I (≥ 99 %) ≥ 250 U/μl, for biotechnology	5,000,000 U/vial**
1.01681.0001 1.01681.0002*	Benzonase [®] endonuclease ELISA kit II for the immunological detection of Benzonase [®] endonuclease	5 plates (8 x 12) plus reagents
*	Note: This Cat. No is valid for USA and Canada only.	
**	This package is only available on request.	
In addition to the above, special package sizes are available on request		

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www.emdchemicals.com

An Affiliate of Merck KGaA,
Darmstadt, Germany

2) Sensitive electrophoretic assay for the determination of Benzonase® endonuclease at low concentrations (0.5 – 5 pg/ml)

Supercoiled plasmid DNA can be converted to its circular, tension free form via a single nucleolytic cleavage or alternatively to its linear form via cleavage of both strands at opposite ends or in an adjacent area. Both of these changes can be easily detected analytically since the three different forms migrate at different speeds during gel electrophoresis. This provides a simple test for the presence of Benzonase® endonuclease in a sample. The detection limit of this test can be effected by other components present in the sample solution, such as chelating agents or highly concentrated salts. Hence, a positive control, in this case a diluted solution of Benzonase® endonuclease, should always be carried out. In addition to this, pure buffer solution should be used as a negative control.

Required reagents and instruments

Instruments: Disposable plastic tubes 0.2 ml/1.5 ml, ice bath, micropipettes 1-20 µl and 20-200 µl with suitable tips, 37°C heating block or water bath, Vortex mixer, gel electrophoresis apparatus and suitable 1 % agarose gel, UV lamp.

Substrate solution: Supercoiled plasmid DNA solution 15 ng/µl (e.g. pBR 322) in 62.5mM Tris-HCl, 6.25 mM MgCl₂, 0.125 mg/ml BSA, pH 8.0. The bovine serum albumin used must be nuclease-free! Storage: -20°C.

Benzonase® endonuclease stock solution: Benzonase® endonuclease (purity grade I, 99 %) ≥ 250 U/µl (exact batch value see certificate of analysis) (Cat. No.: 1.01697 or 1.01695).

Dilution buffer: The same buffer that is used for the enzyme e.g. 20 mM Tris-HCl, 2 mM MgCl₂, 20 mM NaCl, pH 8.0.

Gel loading: 0.5 g/ml sucrose, 1 % bromophenol blue, 100 mM EDTA, pH 8.0.

1) Set up a dilution series (example):

Step 1: 10 µl Benzonase® endonuclease purity grade I + 240 µl dilution buffer ⇒ solution A (1x10⁴ U/ml**) (≥ 99 %) ≥ 250 U/µl* (Cat. No. 1.01695 or Cat No. 1.01697)

Step 2: 10 µl solution A + 900 µl dilution buffer ⇒ solution B (1x10³ U/ml**)

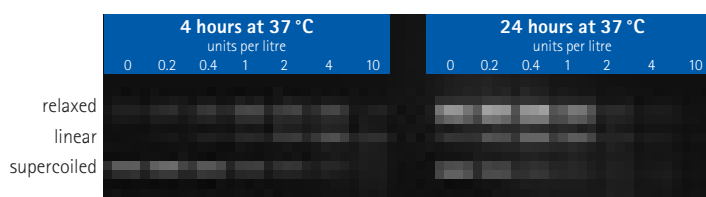
Step 3: 100 µl solution B + 9.9 ml dilution buffer ⇒ solution C (1.0 U/ml**)

* Insert the actual batch value as given on the Certificate of Analysis ** Recalculate according to actual batch values

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5
Solution C (1.0 U/ml)	0.100 ml	-	-	-	-
Standard 1	-	100 µl	100 µl	25 µl	10 µl
Dilution buffer	9.900 ml	100 µl	400 µl	475 µl	490 µl
final concentration**	1 x 10 ⁻² U/ml	5 x 10 ⁻³ U/ml	2 x 10 ⁻³ U/ml	5 x 10 ⁻⁴ U/ml	2 x 10 ⁻⁴ U/ml

- Place 20 µl of substrate solution into each Eppendorf tube. Add exactly 5 µl of sample solution or positive or negative control (dilution buffer).
- Incubate for minimum 4 hours at 37 °C.
- Add 10 µl of sample application buffer and mix. Store at -20 °C until required for electrophoresis.
- Load 15 µl each of sample, positive and negative control onto a 1% agarose gel. Run the gel at a voltage of 1-3 V/cm. Stop the electrophoresis when the bromophenol blue has migrated 4-10 cm.
- Stain the gels for 20 - 30 min in a solution of 0.5 - 1 µg/ml ethidium bromide and evaluate under UV light (photograph). Any residual ethidium bromide can be safely disposed of by means of the new ethidium bromide adsorption columns (Cat. No. 1.02243).

Figure 17: shows the results of a typical assay. Plasmid pBR322 was incubated along with Benzonase® endonuclease purity grade I (99 %) in 100 mM sodium phosphate, pH 6.0, in a dilution series of 0.2-10 units/l corresponding to 0.2-10 pg protein/ml. It can be clearly seen that Benzonase® endonuclease activity of only 0.4 units/l was detected after 24 hours although partial linearization and relaxation can be observed in the negative control. This effect is probably due to traces of endonuclease contamination in the buffer.



3) Semi-quantitative assay for the detection of residual activity of Benzonase® endonuclease

Principle and limitations of this assay

This assay has been designed to detect residual Benzonase® endonuclease activity rather than to provide an exact measure of enzyme activity. The detection limit of this assay is approximately 1 unit/ml. Dilute solutions of Benzonase® endonuclease may be concentrated prior to the assay by ultrafiltration (cut off membrane: $\leq 10,000$ D). The substrate recommended for this assay is sonicated salmon sperm DNA with a molecular weight distribution of 500 to 20,000 base pairs. Low molecular weight cleavage products produced are detected due to the change in absorbance at 260 nm of the residual DNA following precipitation with cold perchloric acid. A standard solution of Benzonase® endonuclease diluted to 2 U/ml is used as positive control, and the dilution buffer is used as negative control. It might be appropriate to include further control samples, e.g. if the sample medium is expected to inhibit the nuclease activity or interfere with the acid precipitation.

Materials

Adjustable pipettes (50 μ l, 500 μ l) with tips, 1 cm quartz cuvettes (ca. 1ml), Eppendorf tubes (1.4 ml)

Instruments

Vortex mixer, Water bath (37 °C), Centrifuge, Ice Bath (0 °C), UV spectrophotometer, Branson Sonifier, Magnetic stirrer

Reagents

DNA stock solution: 2 mg/ml of salmon sperm DNA (e.g. Sigma D1626) is dissolved overnight at 4 °C in 0.2 mM EDTA, pH 8.0 by gentle stirring, and is then sonicated on ice to obtain a homogenous solution. Store 20-50 μ l aliquots at -20 °C. Each new DNA stock solution should be analyzed by agarose gel electrophoresis and compared with the previous batches in a parallel assay.

Dilution Buffer: 50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 0.1 mg/ml bovine serum albumin (BSA)

The BSA must be nuclease-free (Should be tested in a blank system prior to use)

Reaction Buffer: 50 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 0.1 mg/ml bovine serum albumin (BSA), 1 mg/ml sonicated salmon sperm DNA

Benzonase® endonuclease solution: Cat. No. 1.01695 or 1.01697. Store at -20 °C.

Perchloric acid: 4 % (w/w)

Procedure

1. Dilute the Benzonase® endonuclease solution in dilution buffer to give a final activity per volume of 2 U/ml. Keep the diluted solutions on ice.
2. Label two Eppendorf tubes for each sample to be tested, two for the negative and another two for the positive controls. Pipet 450 μ l of the Reaction Buffer into each tube.
3. Add 50 μ l of the sample to the prepared tubes and mix well (Vortex). Add 50 μ l of the Benzonase® endonuclease standard solution to each of the positive control tubes and 50 μ l of dilution buffer to the negative controls. Mix well.
4. Incubate in a water bath at 37 °C for 4 hours (or longer).
5. Transfer the tubes into an ice bath and add 500 μ l of chilled 4 % perchloric acid to each tube. Mix well and leave for 30-60 minutes at 0 °C to completely precipitate DNA present.
6. Centrifuge the tubes for 5 minutes at 15,000 x g (at 4 °C). Carefully transfer the supernatants to fresh tubes.
7. Measure the absorbance of the supernatants in the quartz cuvette against water as blank. The negative controls should not exceed 0.1 AU.
8. Calculate the corrected A₂₆₀ values by subtracting the average negative control value from the samples readings. The corrected absorbance of the positive controls after 4 hours should be higher than 0.5 AU.

4) Chain length of nucleic acids after Benzonase® endonuclease treatment

There are no explicit FDA regulations governing the chain length of nucleic acid contamination that is allowed to be present in pharmaceuticals. The general criteria requested by the regulatory authorities seem to be that fragments need to be smaller than 100 bp. Benzonase® endonuclease, when used under ideal conditions, degrades all nucleic acid sequences down to 3-8 bp oligonucleotide fragments.

The experiments conducted concentrated on showing that Benzonase® endonuclease treatment results in fragments smaller than 100 bp. A real time PCR experiment was conducted using a 96 bp DNA fragment, which could not be amplified post Benzonase® endonuclease treatment. As an additional control, the samples were loaded on to a 10 % TBE Polyacrylamide gel (see Fig. 18). See below for experimental details.

Experimental details

A 96 bp dsDNA fragment of known sequence from *Pseudomonas aeruginosa* was amplified. The resulting fragments were purified, and the concentration determined. 1 Unit of Benzonase® endonuclease (in excess) was added to 1 pg, 100 fg, 10 fg, 1 fg and 0.1 fg of DNA each in 20 µl of PCR buffer. The mix was incubated for 20 minutes at 37° C followed by Real Time PCR using a fluorescence resonance energy transfer (FRET) detection system. This system produces a measurable fluorescence signal, but only when both hybridisation probes are bound to the internal region of the PCR product. Hence the more PCR product present, the stronger the fluorescence signal produced. This allows the amplification to be followed “live” and provides an accurate indication of the amount of DNA present. As an additional control, the samples were loaded onto a 10 % TBE Polyacrylamide gel (see Figure 18).

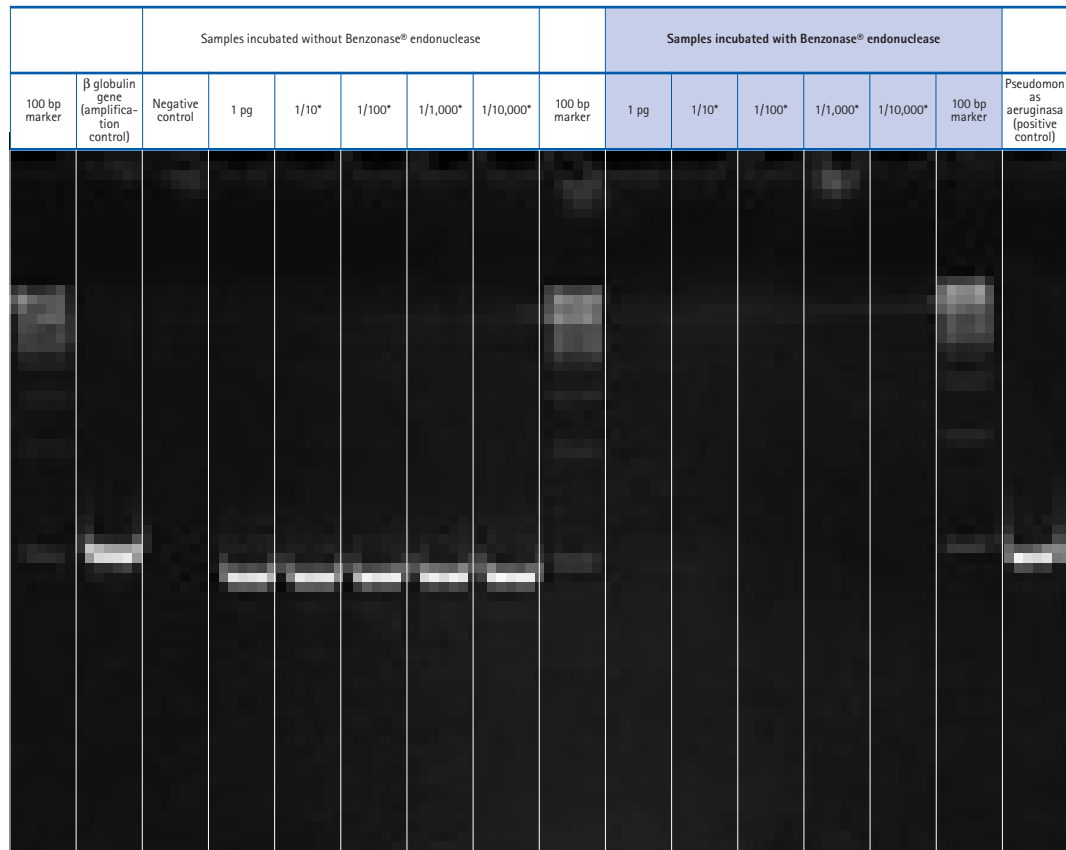
References:

[17] “The kinetics of a sigma subunit directed promoter recognition of *E-coli* RNA polymerase” M. Buckle, I.K. Pemberton, M.A. Jacquet and H. Buc. Journal of Molecular Biology, Vol. 285, No.3,pg.995-964. January 1999

Figure 18: 10 % TBE Polyacrylamide gel of samples following Real time PCR

The lanes containing the samples incubated with Benzonase® endonuclease do not contain any bands, this means that no DNA was amplified. From this we can draw the conclusion that the oligonucleotides produced by Benzonase® endonuclease treatment are below the hybridisation limit.

* Dilution series



5) Detection of Benzonase® endonuclease – Benzonase® ELISA Kit II

This assay detects and quantifies the amount of Benzonase® endonuclease present in samples, thus allowing prove 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 greater 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 %.

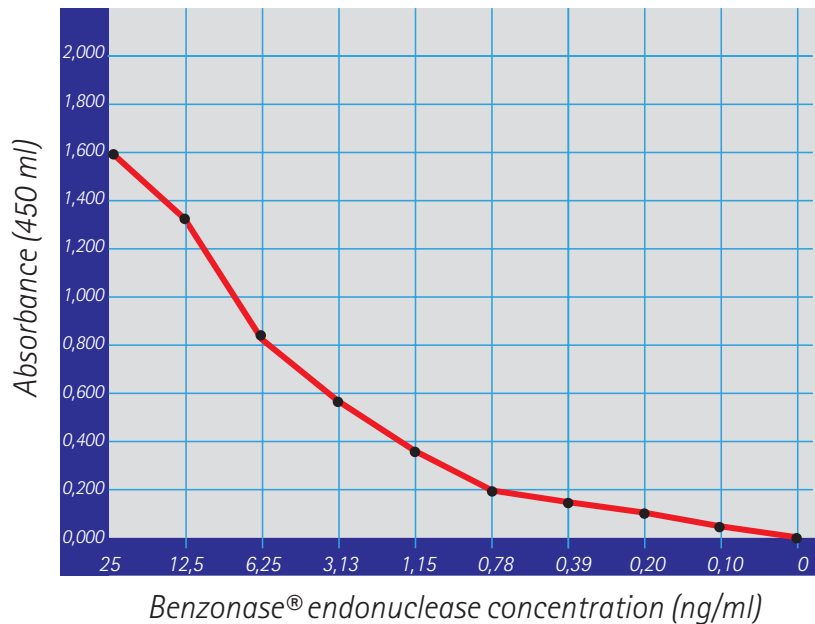
How does the assay work?

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. The reaction is stopped by the addition of 0.2 M H₂SO₄ and the result can be read visually or using an ELISA plate reader at 450 nm.

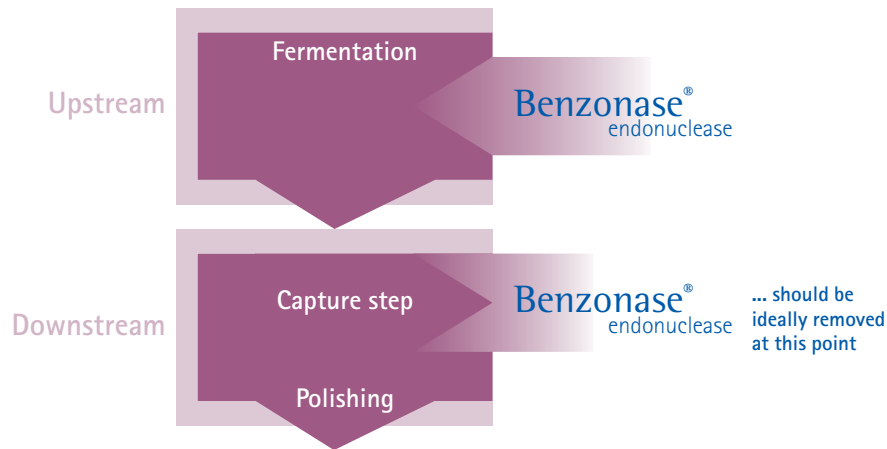
Advantages

- fast and simple to use
- shelf life of 12 months
- low detection limit (0.2 ng/ml)
- single strip plates (8 x 12)

Figure 19:
Exampel of a typical
calibration curve for
Benzonase® endonuclease,
ultra pure grade in the
range of 0.1 to 25 ng/ml



6) Removal of Benzonase® endonuclease



During downstream processing, Benzonase® endonuclease can be separated from the target product using a variety of methods. The easiest of these is a chromatographic separation step, which is often already present in most downstream processing protocols. By adjusting the loading conditions, the target molecule will bind to the column, while Benzonase® endonuclease present either passes straight through or is eluted separately. Depending on the characteristics of the target molecule one of the following can be used: anion exchange, cation exchange, hydrophobic interaction, hydroxyapatite and/or size exclusion chromatography (see tables pages 29, 30).

Anion exchange resins

This probably is one of the most common and cost effective capture steps available. In the example illustrated below (see Fig. 21, page 29) BSA was used as our example target molecule.

Column: Superformance® glass column (5.0 x1.0 cm i.d.) with **Fractogel® EMD DEAE (M)**, bed height 1.3 cm
 Sample: 200 µl Benzonase® endonuclease and BSA (1 mg/ml each in water)
 Sample & equilibration buffer: 50 mM Tris/**100 mM NaCl, pH 8** 5 CV
 Low salt wash buffer: 50 mM Tris/100 mM NaCl, pH 8 5 CV
 High salt wash buffer: 50 mM Tris/**500 mM NaCl, pH 8** 10 CV
 Flow rate: 2 ml/min

CV = column volume

The chromatogram (see Fig. 20), in conjunction with an SDS gel to confirm the identity of the peaks (see Fig. 21, page 29), indicates that Benzonase® endonuclease does not bind to the column but instead passes straight through along with any other impurities present.

Figure 20

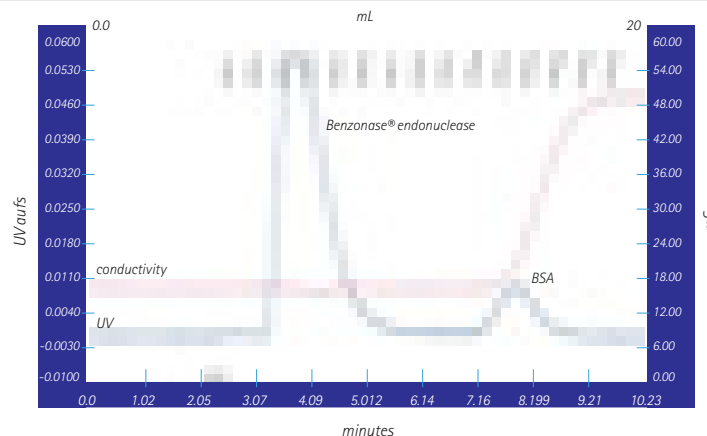
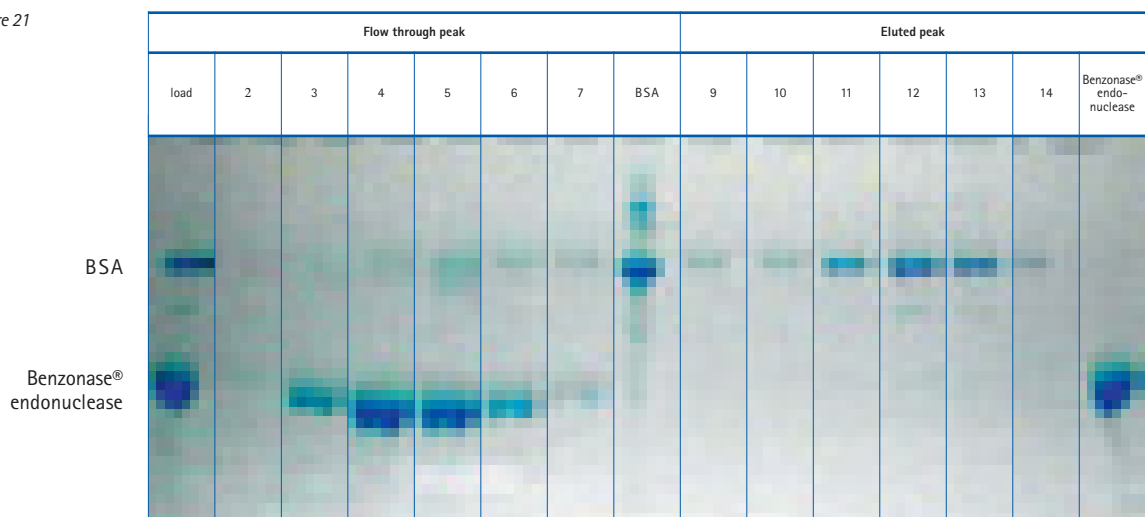


Figure 21



This can be applied to other anion ion exchange resins using a variety of sample and equilibration buffers as illustrated in the table below (Table 2).

Table 2

Fractogel® EMD	pH	Sample & equilibration buffer	Benzonase® endonuclease	BSA
TMAE*	7.0	50 mM Tris / 200 mM NaCl	✗	✗
TMAE	7.0	50 mM Tris / 50 mM NaCl	✗	✓
TMAE	8.0	50 mM Tris / 250 mM NaCl	✗	✗
TMAE	8.0	50 mM Tris / 100 mM NaCl	✗	✓
TMAE	9.0	50 mM Tris / 200 mM NaCl	✗	✓
TMAE	9.0	50 mM Tris / 100 mM NaCl	✗	✓
DEAE*	7.0	50 mM Tris / 200 mM NaCl	✗	✗
DEAE	7.0	50 mM Tris / 50 mM NaCl	✗	✓
DEAE	8.0	50 mM Tris / 250 mM NaCl	✗	✗
DEAE	8.0	50 mM Tris / 100 mM NaCl	✗	✓
DEAE	9.0	50 mM Tris / 250 mM NaCl	✗	✗
DEAE	9.0	50 mM Tris / 50 mM NaCl	✗	✓
DMAE	8.0	50 mM Tris / 250 mM NaCl	✗	✓
DMAE	8.0	50 mM Tris / 50 mM NaCl	✗	✓

Key: ✗ = not bound, ✓ = bound, ✓ = partially bound

* TMAE = Trimethylammoniummethyl, DEAE = diethylaminoethyl; DMAE = dimethylaminoethyl

For more information on Fractogel® EMD resins see www.fractogel.com

These processes can also be applied to non-protein target molecules. For example, Puresyn, Inc. Malvern, PA; uses Benzonase® endonuclease to ensure complete removal of nucleic acids in their viral products. To quantitatively separate Benzonase® endonuclease from the virus the sample is loaded in 50 mM Tris HCl, 100 mM NaCl, 2 mM MgCl₂, 2 % sucrose, pH 8 onto Fractogel® EMD DEAE (M) resin. Any Benzonase® endonuclease present flows straight through while the virus binds to the gel and is eluted by a step gradient using NaCl. Alternatively, for large target molecules, microfiltration can be used to remove Benzonase® endonuclease.

Cation exchange resins

Cation exchange resins can also be used to remove Benzonase® endonuclease. However in comparison to anion exchange columns the range of sample and equilibrium buffers available is narrower, as Benzonase® endonuclease will bind to cation exchange resins under certain conditions (see Table 3).

Table 3

Fractogel® EMD	pH	Sample & equilibration buffer	Benzonase® endonuclease
SO ₃ ⁻	6.0	20 mM phosphate / 100 mM NaCl	✓
SO ₃ ⁻	6.0	20 mM phosphate / 200 mM NaCl	✗
SO ₃ ⁻	5.0	20 mM acetate / 200 mM NaCl	✓
SO ₃ ⁻	5.0	20 mM acetate / 700 mM NaCl	✗
SO ₃ ⁻	4.0	20 mM acetate / 300 mM NaCl	✓
SO ₃ ⁻	4.0	20 mM acetate / 800 mM NaCl	✗
COO ⁻	6.0	20 mM phosphate / 0 mM NaCl	✗
COO ⁻	5.0	20 mM acetate / 40 mM NaCl	✓
COO ⁻	5.0	20 mM acetate / 100 mM NaCl	✗
COO ⁻	4.0	20 mM acetate / 150 mM NaCl	✓
COO ⁻	4.0	20 mM acetate / 400 mM NaCl	✗

Key: ✗ = not bound, ✓ = bound, ✓ = partially bound

Hydrophobic interaction & Hydroxyapatite

Based on the known properties of Benzonase® endonuclease, we would predict that it does not bind to hydrophobic gels. However, we would predict that it binds to hydroxyapatite matrices and can be eluted using ~ 75 mM phosphate.

Literature references:

- [1] US Patent No. 5,173,418
- [2] EP No. 0 229 866
- [3] Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology, 1985
- [4] WHO Expert Committee on Biological Standardization, Forty-seventh Report Technical Report Series, No. 878, 25 ff. 1998
- [5] Barnfield Frej, A.-K., Hjorth, R. & Hammarstrom, A.; Pilot Scale Recovery of Recombinant Annexin V from Unclarified *Escherichia coli* Homogenate Using Expanded Bed Adsorption, in: Biotech. Bioeng. **44**, 922 – 929 (1994)
- [6] Hagen, A. et al.; Use of a nuclease enzyme in the purification of VAQTA*, a hepatitis A vaccine; in: Biotechnol. Appl. Biochem., **23**, 209 – 215 (1996)
- [7] Hagen, A.J., Oliver, C.N. & Sitrin, R.D.; Optimization of Poly(ethylene glycol) Precipitation of Hepatitis A Virus Used To Prepare VAQTA, a Highly Purified Inactivated Vaccine; in: Biotechnol. Prog. **12**, 406 – 408 (1996)
- [8] Hagen, A.J., Oliver, C.N. & Sitrin, R.D.; Optimization and Scale-Up of Solvent Extraction in Purification of Hepatitis A Virus (VAQTA*); in: Biotech. Bioeng. **56**, 83 – 88 (1997)
- [9] Jeltsch, A.; Benzonase® endonuclease – a non-specific nuclease for footprint analysis; in: Merck Biochem. Service 1.95, (W225137) Merck KGaA Darmstadt, Germany
- [10] Gross, M., Wyss, M., Furter-Graves, E. Wallimann, T. & Furter, R.; "Reconstitution of active ocatmeric mitochondrial creatine kinase from two genetically engineered domains" , Protein Science, 1995
- [11] Meinrath, H. G.; in: Biochem. Service 2.93, Merck KGaA
- [12] Beacon 2000 (FP) DNase Activity Detection Kit PanVera Inc., Madison (WI), USA
- [13] Shane, E., Oliver, C. "Scaling-Up, Modelling-Down Case Study: Synagis® (*palivizumab*, *MEDI-493*)"; Process Validation for Manufacture of Biologics and Biotechnology Products, PDA Conference (Berlin, September 2001)
- [14] Reichert, U., Kneips, E., Slusarczyk, H., Kula, M.-R., Thömmes, J., "Isolation of a recombinant formate dehydrogenase by pseudo-affinity expanded bed absorption", Journal of Biochemical and Biophysical Methods; **49**, 533-552 (2001).
- [15] Fernandez-Lahore, H.M., Geilenkirchen, S., Boldt, K., Nagel, A., Kula, M.-R., Thömmes, J., "The influence of cell absorbent interactions on protein absorption in expanded beds", Journal of Chromatography **24**, 873(2), 195-208 (2000).
- [16] Smith, J.G., Liu, X., Kaufhold, R.M., Clair, J., Caulfield, M.J. "Development and Validation of a Gamma Interferon ELISPOT Assay for Quantification of Cellular Immune Responses to Varicella-Zoster Virus" Clinical and Diagnostic Laboratory Immunology, Vol. **8**, No. 5, Pg. 871-879. (2001).
- [17] Buckle, M., Pemberton, I.K., Jacquet, M.A., Buc, H. "The kinetics of a sigma subunit directed promoter recognition of *E.coli* RNA polymerase" Journal of Molecular Biology, Vol. **285**, No. 3, pg.995-964 (1999).
- [18] Huyghe, B.G.; Liu, X.; Sutjipto, S.; Sugarman, B. J.; Horn, M.T.; Shepard, H.M.; Scandella, C.J.; Shabram, P. "Purification of a type 5 recombinant adenovirus encoding human p53 by column chromatography" Human Gene Therapy, Vol. **6**, Issue 11, November 1995, Pages 1403-1416.

Key search words

The world of science is constantly evolving and new scientific literature is being published on a daily basis. To obtain the latest research papers regarding Benzonase® endonuclease please consult our internet site <http://www.benzonase.com/> or alternatively use a scientific search engine such as PUBMED (Internet link section). Some key search words are listed below:

Benzonase

Benzonase + cells

Benzonase + nucleic acids

Benzonase + *E.coli*

Internet links

Benzonase® endonuclease: www.benzonase.com or www.benzonase.de

Fractogel® EMD chromatography resins: www.fractogel.com

Merck Life Science Products: www.merck-lsp.de

Merck KGaA: www.merck.de

EMD Chemicals, Inc. (US affiliate of Merck KGaA): www.emdchemicals.com

Medimmune, Inc.: www.medimmune.com

Puresyn, Inc.: www.puresyn.com

PUBMED - National Library of Medicine's medline and pre-medicine database: www.ncbi.nlm.nih.gov/entrez/query.fcgi

Patent and licence information

Benzonase® endonuclease is covered by several patents, i.e.

US Patent No.5,173,418

EP Patent No.0229866

Nycomed Pharma A/S, Denmark claims all world-wide patent rights to

Benzonase® endonuclease. Merck KGaA is holding an exclusive licence on all patents.

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