

Restriction Endonuclease Troubleshooting Guide

Problem	Probable Causes	Recommended solutions
Undigested or incompletely digested DNA	<ul style="list-style-type: none"> • Nature of DNA The activity of enzyme depends on the nature of DNA substrate - linear or supercoiled, concentration of recognition sequences, origin of nucleotides flanking the recognition sequence. • Impure DNA DNA preparations, especially minipreps, may contain some contaminants such as phenol, chloroform, ethanol, detergents, EDTA, salts, that might partially or completely inhibit the activity of restriction endonuclease. • Lack of recognition sequences • Nonoptimal reaction conditions Enzymatic reaction is characterized by following conditions: incubation temperature, buffer ionic strength and pH, Mg²⁺ concentration. Sometimes the presence of BSA in the reaction mix has the crucial influence on the activity of enzyme, because it stabilizes the enzyme, binds some impurities, prevents the enzyme sorption on the test tube surface. Reaction conditions for each endonuclease are indicated by the producing company on the Technical Data Sheet • Enzyme sensitivity to substrate methylation Each restriction endonuclease is inhibited by the methylation of one particular nucleotide in the recognition sequence. The methylation of other nucleotides in the target sequence might partially or completely inhibit the activity of the enzyme. Most laboratory strains used for plasmid preps contain two site-specific DNA methylases: Dam and Dcm. Enzyme sensitivity to dam or dcm is indicated in the catalog in the product description. • Improper dilution or addition of enzyme 	<p>Verify the activity of enzyme using the substrate indicated in the product description. If the activity is indicated correctly use more enzyme in order to digest the particular substrate DNA.</p> <p>If the enzyme cleaves poorly particular substrate, check the activity of enzyme on unit substrate DNA (λ, Adenovirus-2, pBR322) as well as on the particular DNA mixed with unit substrate DNA. If the activity on unit substrate DNA alone corresponds to that indicated on the enzyme Technical Data Sheet whereas unit substrate DNA mixed with the DNA of interest is digested poorly, repurify the DNA.</p> <p>Check if the DNA sequences recognized by that particular endonuclease are really present in the DNA substrate tested.</p> <p>If it is possible use the reaction buffer recommended by the producer and usually provided with the enzyme. Sometimes the low activity of enzyme might be due to the decay of reaction buffer, in such cases prepare a fresh buffer.</p> <p>Mix DNA of interest and unit substrate DNA, then digest both with the particular enzyme. When the DNA of interest only is digested poorly check if the DNA has any methylation and if the restriction endonuclease used in experiments is sensitive to that methylation.</p> <p>MINOTECH recommends the following dilution buffer: 20mM KPO₄ (pH 7.4), 200mM KCl, 1mM EDTA, 7mM β-mercaptoethanol, 10% glycerol and 0.2mg/ml BSA. The enzyme after dilution with this buffer should be used the same day. In order to store the diluted enzyme the enzyme</p>

	<ul style="list-style-type: none"> • High glycerol concentration Some restriction endonucleases (<i>Nco</i> I, <i>Hpa</i> I) are very sensitive to glycerol concentration in the reaction mix • Partially or completely inactivated enzyme 	<p>storage buffer should be used for dilution. Not dilute the enzyme with 1x reaction buffer. Such dilution might partially or completely inactivate the enzyme, especially if the reaction buffer has low ionic strength and no stabilizing agents. Restriction endonuclease is always added the last to the reaction mix.</p> <p>If the restriction enzyme is supplied in 50% glycerol it should make not more than 1/10 of the final reaction volume.</p> <p>If the reaction conditions and the substrate completely correspond to those indicated on the Technical Data Sheet and the enzyme still gives no cleavage or its activity is lower than indicated that means that due to improper handling the enzyme got inactivated.</p>
<p>Additional bands untypical for the banding pattern of particular substrate</p>	<ul style="list-style-type: none"> • Incomplete digestion • Enzyme “star” activity Some restriction endonucleases under particular conditions (low ionic strength, high pH of the reaction buffer, high enzyme concentration, Mn²⁺ instead of Mg²⁺, organic solvents) relax their substrate specificity - in addition to the normally recognized sequences they begin to cleave the substrate in the other sites. Additional DNA bands in such case should be lower than the expected bands and there should be no additional bands higher than the biggest expected fragment. In the case of star activity, additional bands increase and the typical banding pattern decreases in intensity when more enzyme is added, or the incubation time is prolonged • The enzyme is contaminated with other restriction endonuclease If following all the above recommendations the incorrect banding pattern still persists, especially if smaller than expected additional bands are obtained, the enzyme preparation due to improper handling might got contaminated with the another restriction endonuclease. Sometimes not the enzyme preparation might contain contaminating endonuclease but the reaction buffer, DNA preparation or the stop solution. 	<p>Sometimes it is difficult to distinguish between the untypical banding pattern and incomplete digestion. In the case of partial digestion additional low intensity bands are higher than normal ones and there should be no additional bands below the smallest expected fragment. The bands disappear when the incubation time or the amount of enzymes is increased. Use 5- to 10-fold excess restriction enzyme.</p> <p>In order to avoid the star activity lower excess amount of enzyme should be added (no more than 10-fold), reaction should be performed in the buffer (sometimes designed specially to suppress the star activity) recommended by the producer.</p> <p>Detect second activity by comparing restriction digest pattern to that expected for the DNA used for unit determination.</p>

	<ul style="list-style-type: none"> • DNA preparation tested contaminated with another DNA 	<p>Detect by DNA minus enzyme on gel; digest the DNA with other restriction endonucleases.</p>
<p>Diffuse DNA bands after gel electrophoresis band shift</p>	<ul style="list-style-type: none"> • Impure substrate • Impure enzyme • Protein binding to DNA results in the gel shift in agarose gels 	<p>Incubate the substrate DNA without the enzyme and with the other restriction endonuclease for control.</p> <p>Verify the activity of enzyme using the substrate indicated in the product description. Diffusion bands in such case indicate that the enzyme got contaminated due to improper handling.</p> <p>Incubate the samples before the electrophoresis with 0.1% SDS (final concentration) at 65°C for 10 minutes.</p>
<p>Poor ligation efficiency</p>	<ul style="list-style-type: none"> • Decay of the buffer components ATP and DTT included in the ligation buffer are easily degraded substances. • Restriction enzyme still active in the ligation mix • Nonspecific nuclease contamination Restriction endonuclease, Ligase, or DNA preparation might contain nonspecific nucleases due to improper handling in the lab. • Ligase concentration is to low In the blunt end DNA ligation reactions the concentration of ligase in the reaction mix should be 1.5-7.5Wu/ml. DNA fragments obtained after cleavage with following restriction endonucleases: <i>BseB I</i>, <i>Nru I</i>, are ligated very poorly. 	<p>Repeat the ligation with fresh ligation buffer.</p> <p>If the restriction endonuclease is not heat inactivated at 65°C (thermostable), phenol deproteinization and/or ethanol precipitation of the fragments after the restriction reaction should be performed.</p> <p>Figure out which component of the ligation mix is contaminated by substituting them one by one.</p> <p>In such cases high amounts of Ligase (0.3-0.6Wu/1µg of fragments) and 10% PEG in the ligation reaction should be used. DNA phenol deproteinization after the restriction reaction should be performed.</p>