TECHNICAL MANUAL

Assembly of Restriction Enzyme Digestions

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1. Description

Restriction enzymes, also referred to as restriction endonucleases, are enzymes that recognize short, specific (often palindromic) DNA sequences. They cleave double-stranded DNA (dsDNA) at specific sites within or adjacent to their recognition sequences. Most restriction enzymes (REs) will not cut DNA that is methylated on one or both strands of their recognition site, although some require substrate methylation.

Each restriction enzyme has specific requirements for optimal activity. Ideal storage and assay conditions favor the highest enzyme activity and fidelity. Conditions such as temperature, pH, enzyme cofactor(s), salt composition and ionic strength affect enzyme activity and stability. Two reaction buffers usually accompany each Promega restriction enzyme: The optimal reaction buffer, which is from the 4-CORE® Buffer System (Reaction Buffers A, B, C and D) or is one of the other optimal buffers (Reaction Buffers E–L), and MULTI-CORE™ Buffer. The optimal buffer yields 100% activity for the enzyme it accompanies and serves as the specific reaction buffer for individual digests with that enzyme. The MULTI-CORE™ Buffer, which is designed for broad compatibility with many REs, is provided with enzymes that have 25% or greater activity in this buffer. The MULTI-CORE™ Buffer is useful for digestions with multiple REs because it generally yields higher activity for more enzyme combinations than any other buffer but sometimes results in a compromise in activity. Digests using multiple REs with significantly different buffer requirements may require sequential reactions, with addition of RE buffer or salt before the second enzyme is used.

More information on restriction enzyme compatibilities and buffers for double-digests is available in the **Restriction Enzyme Resource** at: **www.promega.com/guides/**, and in the Promega **iPhone/iPad App**, available on the App Store.

2. DNA Substrate Considerations

Common DNA substrates for restriction enzymes include bacteriophage lambda DNA, bacterial plasmid DNA and genomic DNA. Lambda DNA is a linear DNA that is an industry standard for measuring and expressing unit activity for many restriction enzymes. Intact supercoiled plasmid DNA and DNAs with a large number of the target restriction site require more units of enzyme (two- to tenfold) per microgram than linear DNA if a linear DNA substrate was used in the enzyme activity assay.

PCR Products and Oligonucleotides are relatively small compared to the DNA substrate used in the unit definition. Therefore, when using PCR products and oligonucleotides in a restriction digest, it is essential to consider the molar concentration of enzyme recognition sites and not only the DNA mass. Also, some REs require flanking bases surrounding the core recognition site. This can be a problem when cutting an oligonucleotide or DNA fragment where the recognition site is near the end. When PCR cloning strategies include the use of PCR primers containing an RE site, the primer should be designed with adequate DNA surrounding the core recognition sequence. See the Technical References section at: **www.promega.com/resources/** for information on the ability of specific restriction enzymes to cut PCR products that have restriction sites near the end of the fragment.

DNA Purity is another factor that must be considered. Depending on the purification method and the care taken during handling, the DNA may contain varying amounts of contaminants that affect restriction enzyme digestion. Contaminants may include other types of DNA, nucleases, salts and inhibitors of restriction enzymes. The effect of a contaminant on an RE digest is generally dose-dependent (i.e., inhibitory effects increase with increasing volumes of DNA added to the reaction). Relatively pure DNA is required for efficient restriction enzyme digestion. Contaminating nucleases are usually activated only after the addition of salts (e.g., restriction enzyme buffer) to the DNA. Therefore, appropriate control reactions should always be run in parallel with the restriction enzyme digest (see Section 5).

Buffers containing low concentrations of EDTA (1mM) are often used to protect DNA from nuclease degradation during storage, but EDTA can interfere with restriction enzyme digestion if the final concentration in the reaction is too high. This situation usually occurs when the DNA substrate concentration is low, making it necessary to use a large volume of DNA in the digest. In such cases, it is best to concentrate the DNA (e.g., by ethanol precipitation). Organic solvents, salts, detergents and chelating agents that are sometimes used during DNA purification also can interfere with restriction enzyme activity if they carry over into the final DNA solution. Dialysis or ethanol precipitation with 2.5M ammonium acetate (final concentration before adding ethanol) followed by drying and resuspension can remove many of these substances. Relatively pure DNA is required for efficient restriction enzyme digestion; however, addition of acetylated BSA to a final concentration of 0.1mg/ml can improve the efficiency of restriction enzyme digestion of impure DNA. We recommend that BSA be included in all digests.

3. Enzyme Storage, Handling and Use

Maintain the sterility of reagents used in the RE digestion as well as any tools (e.g., tubes and pipette tips) used with those reagents. Restriction enzymes should be stored in a nonfrost-free freezer except for a brief period during use, when they should be kept on ice. The restriction enzyme is usually the last component added to a reaction to ensure that it is not exposed to extreme conditions. When many similar digests are being prepared, it may be convenient to create premixes of common reagents.

Before assembling the restriction enzyme digestion, thoroughly mix each component, then centrifuge briefly to collect contents at the bottom of the tube. The assembled reaction should be mixed after enzyme addition. Mix all solutions containing restriction enzymes gently to avoid enzyme inactivation.

4. Setting up a Restriction Enzyme Digestion

An analytical-scale restriction enzyme digestion is usually performed in a volume of 20µl with 0.2–1.5µg of substrate DNA and a two- to tenfold excess of enzyme. If an unusually large volume of DNA or enzyme is used, aberrant results may occur. The following protocol is an example of a typical RE digestion.

1. In a sterile tube, assemble the following components in the order listed below.

Component	Volume
Sterile, deionized water	16.3µl
Restriction Enzyme 10X Buffer	2µl
Acetylated BSA, 10µg/µl	0.2µl
DNA, 1µg/µl	1.0µl
Mix by pipetting, then add:	
Restriction Enzyme, 10u/µl	0.5µl
Final volume	20µl

2. Mix gently by pipetting, close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at the enzyme's optimum temperature for 1–4 hours.
3. Add loading buffer to a 1X final concentration and proceed to gel analysis.

   Note: Overnight digestions are usually unnecessary and may result in DNA degradation.

5. Experimental Controls

Experimental controls are necessary to identify, understand and explain problems or inconsistencies in results. The following controls are commonly used in parallel with RE digests: (1) uncut experimental DNA, (2) digestion of a commercially supplied control DNA and (3) no-enzyme "mock" digestion. We also recommend analyzing one or two different size markers in more than one lane per gel (i.e., different locations on the gel).

6. Additional Protocols for Selected Restriction Enzymes

Promega scientists have tested a subset of restriction enzymes for compatibility with rapid digestion (digesting DNA in 15 minutes or less) and direct digestion in GoTaq® Green Master Mix or PCR Master Mix (1,2). The results are shown in Table 1.

A protocol for rapid digestion is provided in Section 6.A, and a protocol for direct digestion of a PCR product is provided in Section 6.B.

Table 1. Compatibility of Restriction Enzymes With Rapid Digestion or Direct Digestion.

Restriction Enzyme	Rapid-Digest-Capable	GoTaq® Buffer-Compatible	Restriction Enzyme	Rapid-Digest-Capable	GoTaq® Buffer-Compatible
AatII	+	—	KpnI	+	+
Acc65I	nt	+	MluI	nt	+
AccI	nt	+	NcoI	+	+
AgeI	+	+	NdeI	+	+
AluI	+	nt	NheI	+	+
ApaI	nt	+	NotI	+	nt
AvaI	nt	+	NsiI	nt	+
BamHI	+	+	PstI	+	+
BglII	—	+	PvuI	—	+
ClaI	+	+	PvuII	+	+
DdeI	+	nt	RsaI	+	nt
DpnI	+	nt	SacII	—	+
DraI	nt	+	SalI	+	+
Eco47III	nt	+	ScaI	+	—
EcoRI	+	—	SpeI	+	nt
EcoRV	+	+	SphI	+	+
HaeIII	+	nt	StuI	nt	+
HincII	nt	+	XbaI	+	+
HindIII	+	+	XhoI	+	+
HpaI	nt	+	XmaI	+	nt

nt = Not Tested; + indicates successful cutting in 15 minutes or less; — indicates unsuccessful cutting. Data and additional information are available in References 1 and 2.

6.A. Protocol for Rapid Digestion of Plasmid DNA

1. To perform a rapid digestion, assemble the following components on ice in 0.5ml tubes in the order listed:

Component	Volume
Sterile, deionized, nuclease-free water	15.8µl
Restriction Enzyme 10X Buffer	2µl
Acetylated BSA, 10µg/µl	0.2µl
Plasmid DNA, 1µg/µl	1µl
Mix by pipetting, then add:	
Restriction enzyme[1]	1µl
Final volume	20µl

[1]The number of restriction enzyme units added to the reaction varies at 5–12 units, depending on the concentration of restriction enzyme used.

2. Mix gently by pipetting, close the tube and centrifuge for a few seconds at maximum speed in a microcentrifuge. Incubate at the enzyme's optimum temperature for 5–15 minutes.

6.B. Protocol for Direct Digestion of PCR or RT-PCR Products in GoTaq® Green Master Mix or PCR Master Mix

The restriction enzymes listed in Table 1 were tested for direct digestion in GoTaq® Green Master Mix or PCR Master Mix using 25µl reactions and a final master mix concentration of 1X.

Note: Even after thermal cycling is complete, the thermostable DNA polymerase retains some activity, which could fill in the sticky ends generated during a subsequent RE digestion. These blunt-ended products can result in reduced cloning efficiency. While adding a restriction enzyme directly to the PCR saves time, purifying the DNA product after digestion might be advantageous to remove small restriction fragments that could interfere with downstream ligation.

1. To perform restriction enzyme digestion, assemble the following components on ice in 0.5ml tubes in the order listed:

Component	Volume
Unpurified PCR product	25µl
Restriction enzyme[1]	0.5µl
Final volume	25.5µl

[1]The number of restriction enzyme units added to the reaction varies at 1–6 units, depending on the concentration of restriction enzyme used.

2. Mix gently by pipetting, close the tube and centrifuge for a few seconds in a microcentrifuge. Incubate at the enzyme's optimum temperature for 2 hours.

7. References

1. Schagat, T. (2007) Rapid DNA digestion using Promega restriction enzymes. Promega Corporation Web site: **www.promega.com/resources/articles/pubhub/enotes/rapid-dna-digestion-using-promega-restriction-enzymes/**

2. Tritle, D. (2006) Activity of Promega restriction enzymes in GoTaq® Green and PCR Master Mixes. Promega Corporation Web site: **www.promega.com/resources/articles/pubhub/enotes/activity-of-promega-restriction-enzymes-in-gotaq-green-and-pcr-master-mixes/**