Using PaqCI™ for Golden Gate Assembly – What Makes it a Special Addition to NEB's DNA Assembly Portfolio?

By Rebecca Kucera, M.S.,
NEB Principal Development Scientist,
New England Biolabs, Inc.

At NEB, we have placed focus on advancements in both the development of new enzymes and maximizing enzyme functionality for Golden Gate Assembly reactions. In that spirit, our Golden Gate Assembly choices now feature a new player: the exciting Type IIS restriction enzyme, PaqCI. In this article, read about how PaqCI (an AarI isoschizomer) can be used for simple to complex 24-fragment assembly, achieving our highest level of efficiency and fidelity yet, and with less concerns regarding the domestication of internal sites due to its 7 base-pair recognition sequence.

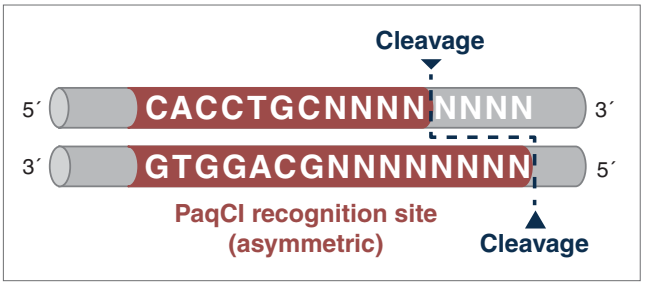
Golden Gate Assembly (GGA) is dependent on Type IIS restriction enzymes that have asymmetric DNA recognition sites and cleave outside of these sequences. NEB currently offers 50 Type IIS restriction enzymes, of which a subset have the necessary favorable characteristics for GGA. Enzymes such as BsaI-HF®v2, BsmBI-v2, and BbsI-HF have been Golden Gate workhorses, as they have historically been featured in published assembly protocols and NEB has extensive experience working with them. During this time, and with input from our customers, we recognized that it would be useful to offer an enzyme with a 7-base recognition site for assembly, along with fully optimized protocols and enzyme recommendations, for assemblies ranging from simple to complex, and at a reasonable price.

The advantage of a Type IIS restriction enzyme with a 7-base recognition site (see Figure 1) is that these sites are less likely to be present in the DNA sequences being assembled, yet they are capable of the full range of assembly complexity that scientists require for their experiments. Through a collaboration between laboratories in NEB's Research, Applications Development, and Production Departments, PaqCI was identified and cloned, and its expression was optimized. A DNA activator for the enzyme was also optimized and protocols were developed for single inserts, as well as simple-to-complex assemblies.

The significance of PaqCI with regards to domestication

Domestication refers to converting any DNA fragment that will be part of an assembly into "Golden Gate-ready" form - flanking the DNA at both ends with the Type IIS restriction sites that will direct the assembly and removing any internal sites for that enzyme that might be present in the DNA and are not tolerated well in GGA. Statistically, a 7-base sequence will appear in any given DNA sequence less often than the 6-base sequence of the more commonly used Type IIS restriction enzymes. Internal sites significantly decrease GGA efficiency because they allow the finished construct to be susceptible to digestion by the restriction enzyme present in the assembly reaction, and could also lead to incorrect and unwanted assemblies.

Figure 1: **Type IIS enzymes recognize asymmetric DNA sequences and cleave outside of their recognition sequence**



This is less of an issue when using Golden Gate for single insert cloning because the overall efficiency for single inserts is high; the desired construct will be assembled even if many of the successfully cloned inserts became linearized and did not efficiently transform. But typically, researchers are using Golden Gate for multiple inserts – and the greater the assembly complexity, the more important the assembly efficiency becomes. For this reason, the presence of an internal recognition site of the chosen restriction enzyme, hinders the assembly.

There are proven methodologies for eliminating internal sites while domesticating DNA sequences: (1) site-directed mutagenesis to eliminate an internal site in advance of the assembly reaction, or (2) designing an assembly junction point right at the internal restriction site with a base change to eliminate the site upon assembly.

However, domestication of a DNA sequence is time consuming, further highlighting the benefit of a 7-base recognition site enzyme, which significantly decreases the probability of internal sites. PaqCI is a 7-base recognition restriction enzyme that has been optimized for Golden Gate Assembly, and is supplied at a concentration that enables use for complex assemblies up to 20+ fragments.

The mechanism of multi-site enzymes and why they benefit from the addition of an activator

Some enzymes have more intricate ways of interacting with their recognition sites in DNA than others. Most homodimeric enzymes, like the standard Type IIP restriction enzymes EcoRI and HindIII, have two identical subunits that bind cooperatively at the symmetric site with each subunit cutting one strand to result in a double-stranded cut. In contrast, multi-site enzymes like PaqCI have a more complex structure and mechanism. It is presumed that PaqCI utilizes multiple subunits to interact with two recognition sites in order to cleave a single target site. To be sure that PaqCI cuts all the sites during Golden Gate Assembly, NEB supplies an inert short oligonucleotide activator containing an extra PaqCI binding site, which functions in *trans* as an activator for PaqCI cleavage (see Figure 2).

By definition, during Golden Gate Assembly, every insert and every destination plasmid has an assembly active DNA fragment flanked by two sites, implying that there is no need for any added sites. But Golden Gate is a very dynamic process, with concurrent cutting and ligating – situations arise where PaqCI binds and cuts sites on different DNA molecules, leaving a remaining site on each molecule to be cut. So having an optimized number of extra sites available in

the form of the PaqCI activator ensures that complete cutting in the assembly reaction occurs. It should be noted that the activator does not get cut or interact in any way with the assembly – it only provides a second binding site that can activate cutting.

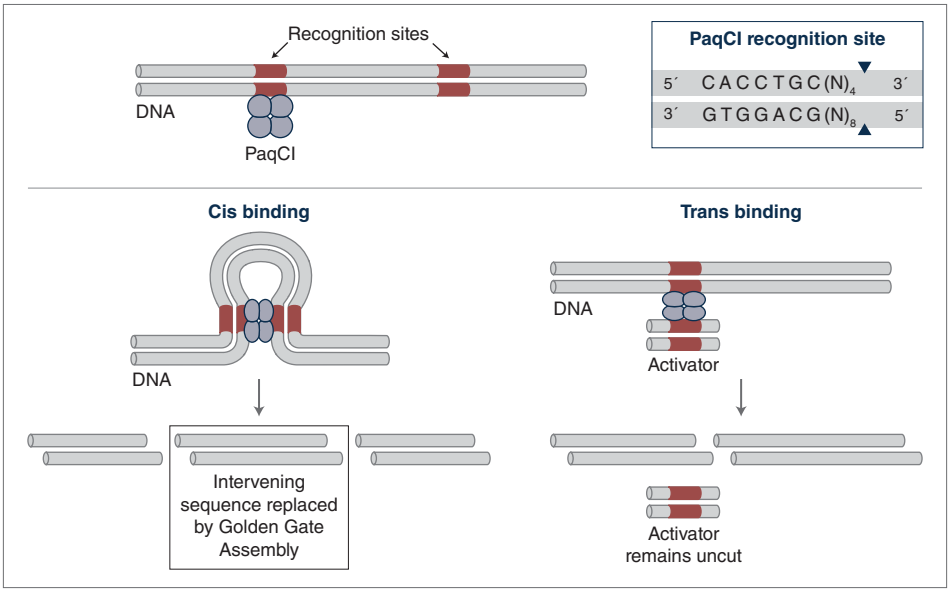
Different levels of complexity call for different levels of PaqCI and T4 DNA Ligase. In addition, PaqCI and activator amounts have been carefully optimized for different assembly complexities. The optimal amount of the activator can be different from what is recommended for a standard restriction digest with PaqCI, where using 1 µl of the enzyme (10 U) requires 1 µl of the activator (20 pmoles). The reason for this is that cutting of DNA in a typical restriction digest, where cut DNA remains cut, is different than what occurs in Golden Gate assembly reactions, where overhangs can sometimes be reannealed and ligated, reconstructing the original recognition site. In the latter case, any one DNA cut site can require being cut more than once throughout the assembly reaction. Because of the dynamic nature of GGA, these regenerated sites translate to less supplementary sites in the form of the activator being needed.

From over a thousand test assembly reactions, NEB researchers have established the optimal amount of PaqCI, activator, and T4 DNA Ligase for everything from simple single insert cloning to a complex 24-fragment assembly (see Table 1).

As assembly reactions increase in complexity, more units of enzyme are required for maximal performance; the range is from 5 to 20 U of PaqCI paired with 200-800 U of T4 DNA Ligase. Recommendations for how much activator to add to each assembly reaction are within a range of 5-10 pmoles. A 20 µM stock of the activator is provided with the PaqCI enzyme.

One note regarding the buffer requirements: while rCutSmart Buffer is the recommended buffer for use in a simple DNA digest with PaqCI, for Golden Gate Assembly, there are better efficiencies achieved by maximizing the PaqCI and T4 DNA Ligase enzyme activities using T4 DNA Ligase Reaction Buffer.

Figure 2: **Presumed mechanism for how the PaqCI activator assures complete cutting via trans binding if needed**



Golden Gate Assembly tools from New England Biolabs

At NEB, we have designed several online tools to help facilitate your Golden Gate workflows.

After designating the DNA fragments for any given assembly, the NEB Golden Gate Assembly Tool can design optimal unique four base overhangs between the inserts that have been independently verified through T4 DNA Ligase fidelity studies to work at high fidelity. It will also automatically check your inserts for the presence of any internal sites that might affect the choice of Type IIS restriction enzyme to direct an assembly, or alert the user to remove such internal sites via domestication. The program will also automatically generate a set of primers for your inserts to add the flanking bases and recognition sites required either for amplicon generation of inserts to be directly used or for pre-cloning purposes. Finally, a report can be generated describing your full assembly with a color-coded graphical read out, your final assembly sequence, and descriptions of each junction between inserts.

In addition, there are also useful programs available under the "Utility" tab within the tool. Those programs can take an uploaded sequence and make suggestions for different desired insert or module design and can also provide you with vetted lists of what overhangs have been found to support high efficiencies and fidelities during Golden Gate Assembly. Together the NEB Golden Gate Assembly Tool makes assembly design easy, even for the first time user! The NEB Golden Gate Assembly Tool is available at **golden-gate.neb.com**.

Learn more about the Golden Gate Assembly workflow and usage guidelines for working with PaqCI by visiting **www.neb.com/R0745**.

For additional information about Golden Gate Assembly, visit **neb.com/goldengate**.

Table 1: **Recommendations for PaqCI Golden Gate Assembly**

Assembly Complexity	PaqCI	T4 DNA Ligase	PaqCI Activator[3]
Single Insert Cloning (10 min 37°C) or Library Prep (60 min 37°C)	5 U	200 U	+ 5 pmoles (1/4 µl 20 µM stock)
Simple to Moderate assembly[1] (2-10 fragments)	5-10 U	200-400 U	+5 pmoles (1/4 ul 20 µM stock)
Complex assembly[2] (11-20+ fragments)	10-20 U	400-800 U	+5-10 pmoles (1/4-1/2 µl 20 µM stock)

1. Based on 5 fragment assembly test system.
2. Based on 24 fragment assembly test system.
3. The activator solution is in a Mg-free buffer for best long-term storage. For short-term working stocks, if desired, dilute an appropriate amount in 1X T4 DNA ligase buffer to achieve more easily pipettable volumes (e.g., a four-fold dilution = 5 µM, 5 pmoles/µl activator.).

www.neb-online.de
NEW ENGLAND BioLabs GmbH
New England Biolabs GmbH, Brüningstr. 50, Geb. B852, 65926 Frankfurt/Main, Germany
Tel: +49/(0)69/305-23140 Toll Free: (Germany) 0800/246-5227 Toll Free: (Austria) 00800/246-52277 Fax: +49/(0)69/305-23149 e-mail: info.de@neb.com