Benchmarks

ical Research to S.H. Address correspondence to Dr. Kenneth R. Peterson, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160-7421, USA. e-mail: kpeter@kumc.edu

Received 26 September 2000; accepted 6 February 2001.

Susanna Harju and Kenneth R. Peterson
University of Kansas Medical Center
Kansas City, KS, USA

Modified Version of pACT-2 that Simplifies Cloning with NdeI

BioTechniques 30:1204-1207 (June 2001)

Since its description in 1989 by Fields and Song (3), the yeast two-hybrid system has proven invaluable for the characterization of protein-protein interactions and the isolation of new proteins that interact with given proteins (for an introduction to many of the aspects of the yeast two-hybrid system, see Reference 1). Although the original method has been modified and many yeast strains have been engineered to improve protein expression and decrease false positives, the rationale remains the same (Matchmaker two-hybrid system, Matchmaker two-hybrid system 2, system 3, and LexA system; Clontech Laboratories, Palo Alto, CA, USA). In addition, variants have been created such that it is now possible to perform a one-hybrid screen to clone cDNA sequences coding for specific proteins that interact with given proteins. However, the pACT-2 contains NdeI restriction sites at position 235 and 6131 in addition to the polylinker. Therefore, pACT-2 is not suitable for directional cloning with NdeI (Figure 1). The companion vectors pAS2 and pAS2-1 each contain a single NdeI site and were thus suitable for cloning of NdeI restriction fragments without modifications.

Because of the number of the clones we wished to construct and the prospect of performing blunt end ligations if we could not use the NdeI site in the polylinker of pACT2, we chose to use in vitro mutagenesis to remove the NdeI sites outside the polylinker. Removal of the NdeI sites outside the polylinker would create a vector that we could use to directionally clone restriction fragments that contain NdeI cohesive ends at the 5' end. Oligonucleotides encompassing each NdeI site were designed such that mutagenesis using the QuickChange™ kit (Stratagene, La Jolla, CA, USA) would result in the change of a single nucleotide, from CATATG to CA CATG, eliminating the NdeI recognition sequence. Oligonucleotides (Life Technologies, Rockville, MD, USA) were used to remove the site at 235 were (top strand) 5'-CTCGGTACTATGCCAT-GATCCAATATCAAAGG-3' and 5'-CCTTGATATTGGATCATGT-AGTACCGAG-3' (bottom strand); the location of the destroyed NdeI site is underlined. An oligonucleotide complimentary to pACT2 sequences 126-149 was used for DNA sequencing to verify the mutation. DNA sequencing was performed by the University of California Davis Division of Biological Sciences DNA Sequencing Facility. Oligonucleotides used to remove the NdeI site at 6131 were (top strand) 5'-GTACTGAGATGTCACCCACTCGGTGGAATACCGC-3' and bottom strand, 5'-GGTATTTCACACCGCATGTG-3'; the location of the destroyed NdeI site is underlined. Verification of the mutation

![Figure 1. Diagrammatic and functional documentation of the important features of pACT2J. (A) Cartoon showing important features of pACT2J. The locations of the NdeI restriction sites (235 and 6131) that were removed from the original plasmid are noted with the word “removed”. The remaining NdeI site in the polylinker has been labeled unique, to emphasize the changes. (B) Results of filter assay for β-galactosidase. Yeast strain SFY526 transformed with two-hybrid plasmid constructs as described in the text was grown on SD media lacking leucine and tryptophan. Colonies from each plate (+ and -) were adsorbed to nitrocellulose filters and assayed for β-galactosidase. The filter marked with a + developed blue staining, which is reproduced in black and white as grey. The filter marked - developed no blue color.](image-url)
was performed by DNA sequencing using an oligonucleotide primer complementary to pACT2 sequences 6033–6051.

Mutagenesis was performed following manufacturer’s instructions for the QuickChange kit. Plasmid pACT2 was sequentially mutated to remove the Ndel site at 235 first, followed by the site at 6131. Following each round of mutagenesis, DNA sequencing was used to confirm the nucleotide change. After both rounds of mutagenesis, restriction digestion was used to verify the unique Ndel site in the polylinker. The resulting plasmid, pACT2J (John and Jodi), was purified by CsCl/ethidium bromide ultracentrifugation and used for cloning. DNA sequencing of the full-length plasmid has not been performed. However, transformation of the plasmid into E. coli and Saccharomyces cerevisiae has been performed.

Plasmid replication and growth of E. coli on LB+amp (100 μg/mL) indicates that the origin of replication and β-lactamase gene product are functional. The ability of the plasmid to replicate and provide LEU2 gene product has been demonstrated by transformation and growth of yeast strain SFY526 on selective media. Figure 1B shows the results of a two-hybrid assay performed to verify the function of pACT2J for yeast two-hybrid screening. Yeast strain SFY526 was transformed with pACT2J containing an insert that encodes bovine filensin (4) and transformants selected on SD [synthetic defined minimal media, yeast nitrogen base without amino acids (Fisher Scientific, Pittsburgh, PA, USA), supplemented with 2% dextrose and amino acids] minus leucine plates. In vivo, filensin assembles in a heteropolymeric manner, with phakosin, and we had previously used the yeast two-hybrid assay to verify interaction between filensin and phakosin (5). To verify the ability of pACT2J to perform in a yeast two-hybrid experiment, yeast SFY526 containing pACT2J-bovine filensin was transformed with pAS2.1 or pAS2.1 containing human phakosin. Transformants were selected on SD lacking leucine and tryptophan plates. Interaction between filensin and phakosin should result in the ability to grow on his− media (not assayed) and the production of β-galactosidase. β-galactosidase was assayed using the filter assay (Matchmaker Two-Hybrid System Manual; Clontech Laboratories). Briefly, nitrocellulose filters are used to lift yeast from selective plates. The adherent yeast colonies are lysed by several rounds of liquid nitrogen freezing, followed by thawing at room temperature. The filters are then incubated with buffers containing X-Gal. Positive colonies are revealed after several hours of incubation. As expected, Figure 1B shows that yeast-harboring plasmids encoding both filensin and phakosin express β-galactosidase, while the negative control does not.

REFERENCES

We are willing to provide aliquots of the plasmid to interested investigators using the computer shareware-derived distribution method, postcardware. Interested parties should send a local origin postcard to the authors at the address listed. In return, plasmid DNA spotted on filter paper will be promptly mailed. This report was supported by National Institutes of Health grant no. EY08747 to P.G.F. Address correspondence to Dr. John F. Hess, Dept. of Cell Biology and Human Anatomy, 1 Shields Ave., University of California, Davis, CA 95616-8643, USA. e-mail: jfhess@ucdavis.edu

Received 18 September 2000; accepted 20 February 2001.

John F. Hess, Jodi T. Casselman, and Paul FitzGerald
University of California
Davis, CA, USA