Genomics 97 (2011) 321–325

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Methods

Efficient generation of gene knockout plasmids for *Dictyostelium discoideum* using one-step cloning

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ARTICLE INFO

Article history:
Received 14 January 2011
Accepted 3 February 2011
Available online 19 February 2011

Keywords:
Gene deletion
Vector
Plasmid
PCR
Dictyostelium discoideum

ABSTRACT

The amoeba *Dictyostelium discoideum* is a well-established model organism for studying numerous aspects of cellular and developmental functions. Its rather small (~34 Mb) chromosomal genome and the high efficiency of gene disruption by homologous recombination have enabled researchers to dissect various specific gene functions. We describe here the use of one-step cloning for the fast and efficient generation of deletion vectors that are produced in a one-step reaction by inserting two PCR products into an organism-specific, generic acceptor system. This worked efficiently for all 16 tested constructs directed against genes in the amoeba *Dictyostelium discoideum*. Saving cost and time, the used protocol represents a significant advancement in the generation of such plasmids compared to the conventionally applied restriction enzyme/ligation approach. Using appropriate selection markers, similar systems could also be useful in other organisms, where genes can be knocked out by homologous recombination.

1. Introduction

Homologous recombination denotes the exchange of nucleotide sequences between identical or nearly identical DNA strands. It is a key mechanism both for the maintenance of genetic information and likewise for the generation of genetic variation by allowing for novel combinations of DNA sequence information. The natural mechanism of recombination is routinely used to exchange or disrupt genes by genetic engineering.

Genes can be disrupted or deleted by various constructs, the design of which essentially depends on the idiosyncrasy of the model organism of interest. Usually, a deletion vector requires the presence of two fragments that are homologous to parts of the gene of interest (right and left arms) and that surround a selection marker. If such a construct is transformed in the respective organism, two homologous recombination events can lead to the generation of a largely deleted gene [1–3] (Fig. 1A). Since this is a rare event, however, the selection marker is required as it allows to propagate only those cells that harbor the transgene construct, whose integration at the homologous site has to be confirmed independently.

Among eukaryotic organisms, there is considerable variation in the length of the arms that need to be included to achieve the desired gene disruption. The baker's yeast *Saccharomyces cerevisiae* likely represents the least demanding where stretches as short as 20 nucleotides (nts) are sufficient [1]. Other species require considerably longer sequences of several hundreds of nucleotides up to the kilobase (kb) range, as it is the case for example in mice [3], or the amoeba *Dictyostelium discoideum* [2] that we are interested in. Due to this requirement, the gene deletion constructs for such organisms cannot be readily created from short oligonucleotides, as it is the case for baker's yeast. Instead, they need to contain two longer arms that usually are generated in the form of two PCR products of appropriate length that correspond to regions of the gene of interest. Conventionally, the two arms are then cloned separately in a cloning vector, sequenced, and inserted one after the other in another vector that contains the selection marker. This series of re-cloning events relies on the presence of compatible restriction enzyme recognition sites, which allow for the directed insertion by using DNA ligase. Thus, for organisms where the homology arms are long, as in mice or amoebae, a stepwise analysis of a number of clones has to be performed for up to four consecutive cloning steps. Here we describe an alternative strategy for the generation of gene knockout vectors for the amoeba *Dictyostelium discoideum*, in which the required arms can be shuttled in the destination vector in a one-step reaction in vitro (Fig. 1B–E).

2. Materials and methods

2.1. Generation of PCR products from genomic DNA of D. discoideum

Genomic DNA was isolated as described previously [4]. Primer sequences were designed as outlined in Fig. 2. Whenever possible, the recognition sequence of the same restriction enzyme was added to the LA1 and RA2 primers. For use in the pKOSG-IBA-dicty1 vector, suitable restriction enzymes are for example Bgl I, Bpu 1102 I, Eco RI, Kpn I, Pst I. Among these any can be chosen whose recognition sequence does not

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doi:10.1016/j.ygeno.2011.02.001

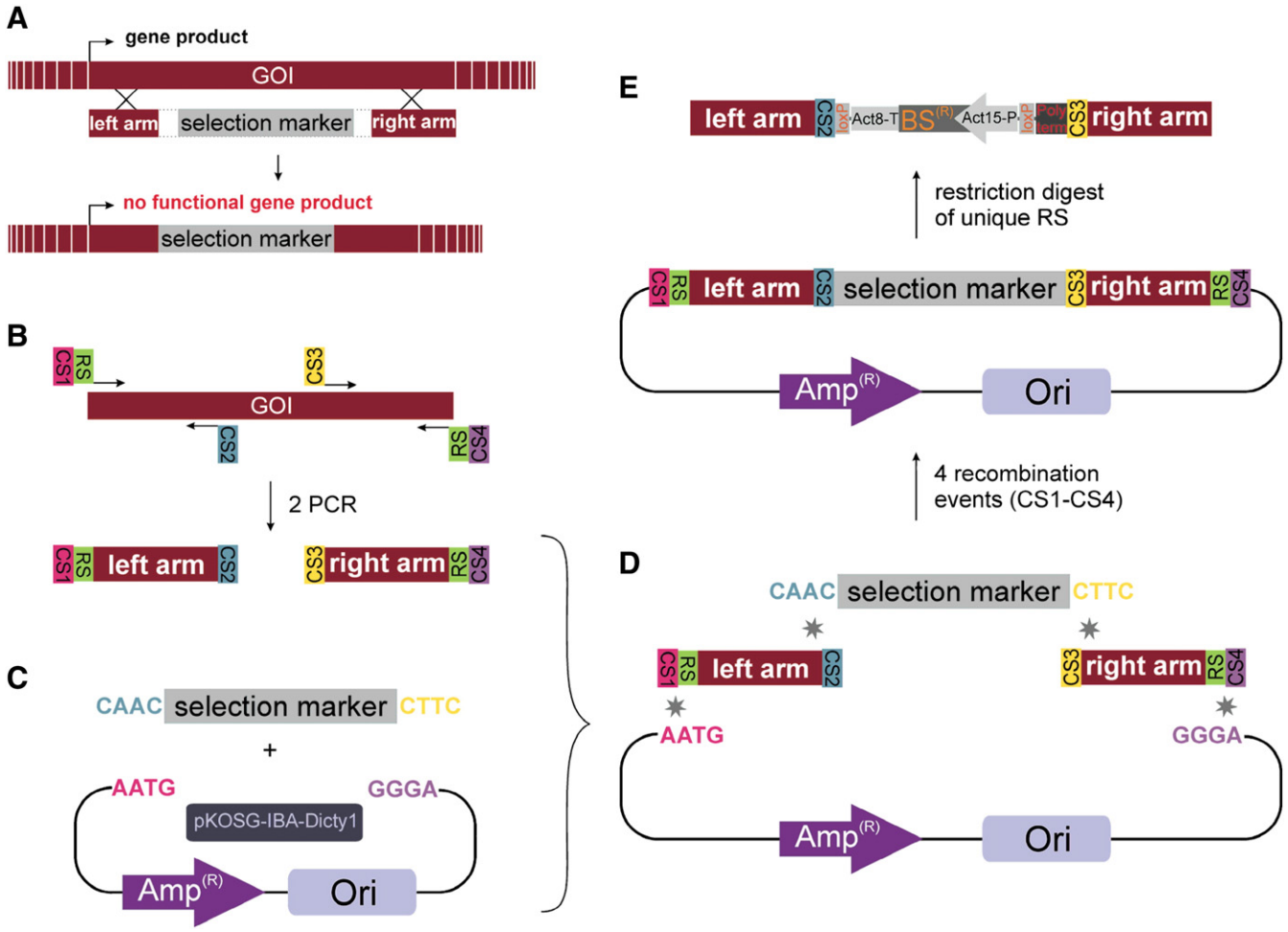

Fig. 1. Gene deletion by homologous recombination. (A) Two recombination events between a gene of interest and a disruption construct featuring two parts of that gene (left and right arm), which surround a selection marker can lead to a disrupted and partially deleted gene. (B–E) Generation of gene deletion constructs by one-step cloning. (B) Left and right arms are created by PCR, using specific primers that all contain at their 5′ ends the StarCombinase binding area (omitted for clarity), the specific, appropriate combinatorial sites (CS) and a unique restriction site (RS) as indicated. (C) The generic acceptor system pKOSG-IBA-dicty1 features, next to the StarCombinase binding area (omitted for clarity), four combinatorial sites (CAAC, CTTC, AATG and GGGA) that flank the selection marker and a vector backbone with a resistance gene (Amp^R) and an origin of replication (ORI) for bacterial propagation. (D) A reaction between the DNA fragments from (B) and (C), mediated by StarCombinases (stars), leads to the desired gene deletion construct in a one-tube reaction. (E) An enzyme recognizing the RS is used to initially analyze plasmids from bacterial clones and can also be used to generate the linear gene disruption fragment required for transformation of eukaryotic cells (cf. A). The restriction enzymes used are listed in Table A.1.

occur in either of the selected arms for homologous recombination. The resulting primer sequences used here are summarized in Table A.1. For some primers, we made use of naturally occurring restriction enzyme recognition sequences within the gene, for example, RA2 of drnA KO I. In general, PCR amplified 400–700 bp segments of *Dictyostelium* genomic DNA for use as homology arms. Standard PCR reactions were carried out using a mixture of Pfu and Taq DNA polymerases (1:2). As a general protocol we have used 10–100 ng genomic DNA of the *Dictyostelium*

primer name	combinatorial site	sequence of primer (5'→3')[a]
LA1	CS1	AGCGCGTCTCCAATG - **unique RS** - forward sequence left arm
LA2	CS2	AGCGCGTCTCCGTTG - reverse sequence left arm
RA1	CS3	AGCGCGTCTCCCTTC - forward sequence right arm
RA2	CS4	AGCGCGTCTCCTCCC - **unique RS** - reverse sequence right arm

Fig. 2. Primer design for cloning with pKOSG-IBA-dicty1. RS denotes the unique restriction enzyme recognition sequence site that is added to the primer for plasmid analysis and cloning in *Dictyostelium discoideum*. The StarCombinase binding areas are printed in light blue and the combinatorial sites are shown with the color code used in Fig. 1.