TARGETING VECTOR CONSTRUCTION BY YEAST ARTIFICIAL CHROMOSOME MODIFICATION PETER J. MURRAY pdf

1: - NLM Catalog Result

Targeting Vector Construction by Yeast Artificial Chromosome Modification Peter J. Murray Summary Mouse yeast artificial chromosomes (YACs) are useful platforms for.

Murray,1 and Elaine I. The platelet- activating factor receptor PAFr has been suggested as a portal of entry. Uptake of the pneumococcal vacuole involved clathrin, and half the bacteria proceeded into vacuoles marked by Rab5 and later Rab7, the classical route to the lysosome. The integrity of body compartments is maintained in part by many respiratory pathogens that externally display phospho- the boundaries of epithelial and endothelial cells. Invasive rylcholine on their surfaces 22, 24, Most bacteria ence was absent in untransfected epithelial cell lines 5. Ring enter cells by subverting host cell membrane trafficking and et al. Transparent colony variants bearing more phospho- compartment suited for bacterial multiplication How- rylcholine interacted with brain microvascular endothelial cells ever, a few significant human pathogens do not multiply intra- three- to fivefold better than opaque variants. One example is Streptococcus pneumoniae, the lead- vacuole that can then either recycle back to the cell surface or ing gram-positive pathogen causing pneumonia, sepsis, and fuse with lysosomes to terminate signaling 3, 4. When pneu- meningitis Pneumococci translocate across human endo- mococci bind PAFr, trafficking of the vacuole has been shown thelial cells without intracellular multiplication The not only to recycle to the apical surface but also to transmi- mechanism of this intracellular routing is unknown. Pneumococci bind to the PAFr via cell wall vesicular trafficking system. Utilization of PAFr as a mechanism of endocytosis This process also uncouples G-protein sig- bacterial entry into human cells was first described for pneu- naling and down regulates the response to ligand-bound mococci 5 but is now recognized as a portal of invasion for GPCRs 4. Pneumococcal uptake is consistent with this con- cept, since classical G-protein signaling is absent upon ligation of PAFr by pneumococci 5, 9. For invasion assays, infected cells were washed and incubated tin-mediated event. Cells were counted, PAFr and, if so, what host cell mechanisms the bacteria use to lysed with 0. The bacterial strains used were T4R, , and min. Trypan blue exclusion was quantified by counting a minimum an unencapsulated derivative of the clinical strain of S. Alternatively, 4 10 , and D39X, an encapsulated, stable bioluminescent derivative of D39 Pneumococci with integrated plasmids were grown in the plated on mm tissue culture dishes for 24 h. The pellet was resuspended in high-salt buffer 20 mM 1: Subsequently, the cells were incubated with as the nuclear extract. Protein concentrations were determined using the Bio- either monoclonal mouse antibody against TEPC 1: To confirm Texas Red goat anti-mouse antibody 1: To label the Inc. The lasers permitted the imaging of Generation of mice lacking the PAFr. The PAFR is encoded on a single exon, green emission, nm , red emission, nm , and far red emission, nm; and the knockout strategy was designed to delete of 1, bp of the coding pseudocolored blue, TO-PRO-3 fluorochromes, respectively. Scanning was per- region. Single gene carried on a yeast artificial chromosome YAC clone as described in detail optical sections 0. Primers were designed to counting at least rBCEC6 cells. Yeast were transformed and 2 mM L-glutamine Cellgro. BamHI was used V53D 0. Recombinants were selected on LB plates with zeocin. This cassette was be internalized. Genomic Invasion and adhesion assay. Five of ES clones T4R 6. Chimeric mice were iden- same mechanisms, unencapsulated variants adhere more, making quantitation in tified by coat color and used for germ line transmission of the mutant allele. For adhesion assays, infected cells were washed, trypsinized, and were genotyped using the PCR-based strategy depicted in Fig. Colocalization of PAFr and pneumococci. PAFr labeled with Alexa tagged secondary antibody appeared green. Colocalization was indicated by yellow in the merged images. Blue staining shows the location of the nucleus. At 21 h, blood and cerebrospinal fluid CSF samples were were imaged using the Xenogen IVIS camera to document differences in the collected, and bacterial titers were determined minimum level of detection, course of invasive disease. The nonvisual None 2. Confocal microscopy studies Fig. Upon infec- cell culture systems has indicated that the presence of the tion with S. An overlay of the two images demonstrated able at http: Colocaliza- colocalization of the signals

consistent with close apposition of tion was independent of G protein signaling by PAFr, since the bacteria and endocytic vesicles containing the PAFr. Interaction of pneu- was not toxic trypan blue uptake of 3. S2 in the mice. Taken together, the colocalization and breeding capacities. To detect translocation in the lung, data of Fig. The C-terminal region minescent imaging example shown in Fig. Escape of bacteria from the lung into tant, prevents GPCR targeting to clathrin-coated pits 4. This suggested that translocation from the lung tion 4. Despite adequate adherence, S. In contrast, mice Fig. This indicates adherence, cotransfection with pairs of constructs bearing dys- VOL. Effect of absence of PAFr on the course of pneumonia and meningitis in vivo. Data for individual mice are shown. MAP kinase activation during invasion. Phosphorylation was detected after 15 min Fig. Colocalization with Rab proteins. Rab proteins belong to the group of Ras small GTPases Different Rab proteins are located in specific intracellular membrane compartments where they regulate endocytotic pathways. Some of the crucial Rab proteins are Rab5, which is involved in early endocytosis, Rab7, which is found in the late endosome, and Rab11, which regulates recycling This was confirmed using the alternative early and late endosomal markers EEA1 and Lamp1, respectively see Fig. S2 in the supplemental ma- terial. This suggested that at least half of the entering infection and is required for PAFr-dependent bacterial invasion. Blue staining defines the nucleus. The assays were repeated a minimum of three times for each condition. Cells were then infected as before and lysed, and ERK phosphorylation was measured by Western blotting. Mutations In vitro S. This process is dependent on the PAFr prevented PAFr targeting to clathrin-coated vesicles elimi- and involves molecular mimicry of PAF by the bacterial cell nated pneumococcal invasion. Colocalization of the bac- from that involved in endocytosis initiates signaling by the teria with PAFr, as shown in this study by confocal microscopy, MAP kinase pathway. Consistent with this, the pneumococcus-induced PAFrâ€" the blood-brain barrier, so as to develop meningitis. A more stable than a non-PAFr-dependent translocation route. Since i activation of MAP kinases was experienced diminished growth of pneumococci in the lungs required for pneumococcal uptake, ii blocking PAFr-medi- and a delayed mortality from pneumonia compared to WT ated MAP kinase phosphorylation did not prevent bacterium-mice Progression to both destinations endocytosis Consistent with both of these steps, exposure of and are involved in vesicle budding, motility, and fusion Further progression through the late blocked by the interruption of clathrin. Our transfection ex- endosome to the lysosome involves Rab7. Colocalization of pneumococci and Rab proteins. Immunostaining for endosomal markers was performed by Alexa tagged secondary antibody green , and bacteria were detected with Texas Red-tagged secondary antibody red by confocal microscopy. Staining of anti-Rab11 is shown at 15 min, staining of anti-Rab5 is shown at 30 min, and staining of anti-Rab7 is shown at 60 min. Colocalization of markers is indicated by yellow. Few vacuoles acquired Rab11, consistent with known recycling of only a small portion of the bacteria back to the TABLE 2. Colocalization of pneumococci with Rab5, Rab7, apical cell surface. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of machinery subverted by pneumococci to drive PAFr-contain- Streptococcus pneumoniae. Uptake and survival of bacteria required the scaffold of the Salmonella-containing vacuole. Expanding roles for beta-arrestins toward transcytosis of viable bacteria. Organ-specific models of Streptococcus pneumoniae disease. Bhuvanendran for technical Ishii, assistance and J. Improved host defense against pneumococcal pneumonia in platelet-activating factor receptor-deficient mice. Molecular analysis of a novel bidirectional pathway.

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2: Yac protocols - literatura obcojÄ™zyczna | KsiÄ™garnia BookMaster

Mouse yeast artificial chromosomes (YACs) are useful platforms for manipulation of targeting vector design and construction, particularly in circumstances where polymerase chain reaction-mediated.

Day and Glyn Stacey, Volume 2, Structure Determination, edited by Sylvie Doublie, Methods and Protocols, edited by Ezio Rosato, Target Discovery and Validation Reviews and Protocols: Quantitative Proteomics, edited by Salvatore Sechi, Methods and Protocols, edited by Wolfram Weckworth, Methods and Protocols, edited by Fernando Vivanco, Lansing Taylor, and Jeffrey Haskins, Methods and Protocols, edited by Pamela C. Methods and Protocols, edited by Elena Hilario and John. Methods and Applications, edited by Kevin Strange, Verma and Alan Trounson, Volume 2, edited by Kan Wang, Volume 1, edited by Kan Wang, Methods and Protocols, edited by Sean P. Methods and Protocols, edited by Charles S. Henry, Gene Mapping, Discovery, and Expression: Methods and Protocols, edited by M. Bina, Methods and Protocols, edited by James D. Stockand and Mark S. Dennis Lo, Rossa W. Allen Chan, Designs and Protocols, edited by Vladimir V. Isolation and Characterization, edited by Kursad Turksen, Nelson, Epidermal Growth Factor: Methods and Protocols, Patel and Paul J. Darby and Tim D. Hewitson, Nuclear Reprogramming: No part of this book may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording, or otherwise without written permission from the Publisher. All papers, comments, opinions, conclusions, or recommendations are those of the author s , and do not necesarily reflect the views of the publisher. This publication is printed on acid-free paper. Jennifer Hackworth Cover design by Patricia F. Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Humana Press Inc. For those organizations that have been granted a photocopy license from the CCC, a separate system of payment has been arranged and is acceptable to Humana Press Inc. The fee code for users of the Transactional Reporting Service is: Printed in the United States of America. QU Y12] I. Methods in molecular biology Clifton, N. YAC Protocols was first produced to address the huge demand within the research community for a lab-based text that described in detail the wide range of uses for large insert yeast artificial chromosome YAC DNA clones. In doing this, the original editor, David Markie, and the many different contributors who provided descriptions of the protocols they used and developed, did a magnificent job. Indeed many of the techniques described within the first edition require little change and have stood up admirably to the test of time. Since the first edition, the use of YACs has proved invaluable for addressing a wide range of new biological problems ranging from those of basic biochemistry to assisting in the mapping and sequencing of the human genome. The requirement for a second edition of YAC Protocols was prompted by a number of major advances in biology since the publication of the first edition. These advances have included the sequencing of the human genome, and the genomes of a wide variety of other organisms, and the increased use of transgenic animals for understanding the molecular basis of human and animal disease. In addition, since the publication of the first edition, the use of YACs for a variety of different applications have been replaced by the use of other large insert cloning vectors such as P1 and bacterial artificial chromosomes PACs and BACs. For this reason, YACs are still the cloning vehicle of choice when studying the characteristics of genomic fragments greater than kb in length. The intention of this second edition of YAC Protocols is not to completely replace the first edition, whose protocols, in many cases, are still relevant today, but to provide a much needed update on the new techniques currently being employed and to help redefine and illustrate the important roles still to be played by YAC technologies in the postgenomic age. Both Chapters 2 and 3 have been written by Cecilia Sanchez and Michael Lanzer and describe how YAC libraries can be generated from the genomes of novel species and pathogens whose genome sequences have yet to be sequenced Chapter 2 and how, in the absence of extensive sequence data required for the design of PCR primers, these libraries can be screened for selected YAC clones by filter hybridization Chapter 3. Chapter 4, by Sylvia Vasiliou and John Quinn, describes how an isolated

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YAC clone can be characterized further using restriction digestion, Southern blotting, nucleic acid hybridization, and PCR analysis to confirm the identity of the clone and to determine its integrity. Gaining access to, and analyzing, the huge wealth of mapping and sequence information currently available via the internet was an option almost undreamed of immediately after the publication of the first edition of YAC Protocols. In Chapter 5, Kerry Miller and Scott Davidson give a basic overview of how genomic sequence can be accessed and how this sequence can be rapidly analyzed using freely available and user friendly internet tools. In Chapter 7, Sanbing Shen describes how YAC clones can be altered to change different auxotrophic markers, a process known as retrofitting, and to allow amplification of the copy number of YAC clones within yeast cells. This ability provides advantages when attempting to isolate larger quantities of YAC DNA for a range of different procedures such as the production of YAC transgenic animals. In Chapter 8, Gabriela Loots describes how YAC clones can be conditionally altered by the engineering of loxP sites flanking target sequences within the clone. The flanking of these target sequences with loxP sites allows for their excision in vivo once the YAC clone has been successfully introduced into the genome of an animal transgenic for an inducible Cre gene. Natalay Kouprina and coworkers describe a method that is likely to be the most important development in YAC techniques since their inception. In Chapter 9, Dr. Kouprina describes how novel YAC clones of a defined size and genomic content can be produced using homologous recombination TAR cloning in yeast. This technique has become especially relevant in recent years as a result of unparalleled access to multiple genome sequences via the internet. Furthermore, TAR cloning of YACs will promise to remove the specter of Preface vii chimerism that has represented one of the main perceived obstacles to the more widespread use of YAC clones. One of the main obstacles centers on difficulties encountered in the use of long range hi-fidelity PCR to isolate the long stretches of homologous sequences required to ensure the production of an efficient targeting vector. These problems include the insertion of replication errors that, despite what their name suggest, still affect hi-fidelity polymerases. These replication errors reduce recombination efficiencies and may even mutate the wrong part of the protein a problem that would compromise the development of a conditional knockout model. Furthermore, the frequent inability to successfully amplify many long sequences owing to the presence of repetitive DNA is common. The ability to develop these vectors will greatly enhance the success of gene targeting in mouse embryonic stem cells. The use of transgenic animals has become an important tool in understanding the role of genes in supporting health. Our added ability to produce transgenic animals using YACs has enhanced our understand of how large genes, or clusters of genes, and their regulatory elements contribute to the development and normal physiology of organisms and how changes within components of these genes can predispose individuals to disease. In Chapter 13, Alasdair MacKenzie describes a protocol that allows for the efficient recovery of intact YAC DNA and its subsequent microinjection into the pronucleii of one-cell mouse embryos. Moreira and coworkers, who have devised an efficient method of introducing intact YAC DNA into the mouse genome using intracytoplasmic sperm injection. This ground-breaking method complements, and may eventually supersede, viii Preface the use of pronuclear injection in the production of YAC transgenic animals. The introduction and analysis of large genomic fragments in highly differentiated primary cell types such as those of the nervous system or the immune system has the real potential of facilitating a better understanding of the roles of genes in maintaining the differentiated phenotype and physiology of these cells. Furthermore, the use of large human genomic fragments to essentially humanize these cells will greatly aid in the design of more specific and efficacious drug therapies. However, getting these large genomic fragments into differentiated cell types and maintaining these cell types indefinitely has been problematic. Carl Anthony Blau and Kenneth Peterson have addressed many of the obstacles preventing the analysis of large genomic fragments in differentiated cell types. This has been achieved by first producing transgenic animals with YACs, deriving differentiated transgenic cells from these transgenic lines and immortalizing these lines using a novel strategy based on the introduction of crippled retroviruses. A detailed protocol outlining the procedures involved is described in Chapter YACs will continue to play a significant role in genome mapping as, although many

genomes have been sequenced, many more important genomes, such as those of the myriad of pests and pathogens that still affect a distressing proportion of the human population, remain to be mapped. An essential aspect of mapping the geography of any genome is to understand the chromosomal location of particular genes and markers within that genome. A technique known as fluorescence in situ hybridization has been critical in developing detailed maps of different genomes. In addition, in Chapter 17, Marcia Santos et al. I am extremely grateful to all the authors who have enthusiastically provided these cutting edge protocols and I would like to acknowledge their help and support in realizing this new edition of YAC Protocols. However, many of the protocols described in this volume specifically address these problems. By gathering these protocols into one volume and demonstrating the enormous potential use of YAC technolo- Preface ix gies in a variety of different research situations, we hope that the use of YAC technology will continue to be considered by the wider research community as a viable tool in understanding the role of genetics in maintaining health, promoting pathogenesis, and conferring susceptibility to disease. Alasdair MacKenzie Contents Preface Sanchez and Michael Lanzer Noskov, and Vladimir Larionov Anthony Blau and Kenneth R. However, this approach requires a familiarity with basic procedures derived from yeast genetics, and lack of experience can be a barrier to researchers with backgrounds limited to bacterial and mammalian culture systems. To address this issue, this chapter introduces and discusses the genetic markers and selection systems used in the genetic manipulation of yeast, with particular reference to yeast artificial chromosome YAC cloning and manipulation. Detailed descriptions of culture media are provided, allowing for the growth and selection of standard YAC strains, as well as for specialist applications. Yeast genetic markers; yeast selection; culture media. Introduction The genetic markers used in yeast, and in yeast artificial chromosome YAC cloning, are largely defined by the manipulation of growth media. This chapter, apart from providing recipes for media formulation, also contains a brief introduction to genetic markers in yeast that are relevant to YAC cloning. It is hoped that this will provide an adequate understanding of the media that are used for particular applications, and allow adaptation of media for individual requirements. Auxotrophic Markers In contrast to bacterial cloning systems, where the use of dominant antibiotic resistance determinants is common, selective markers used in YAC manipulation are based mainly on complementation of biosynthetic mutations. Methods in Molecular Biology, vol.

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3: USA - Conditionally amplifiable BAC vector - Google Patents

Abstract. Mouse yeast artificial chromosomes (YACs) are useful platforms for manipulation of targeting vector design and construction, particularly in circumstances where polymerase chain reaction-mediated amplification of targeting arms proves fruitless or the cloned DNA is inherently unstable.

BACs are preferred because individual DNA fragments are maintained stably in a single copy vector in the host cells, even after or more generations of serial growth. BAC or pBAC vectors typically accommodate inserts in the range of approximately 30 to kilobase pairs. A widely used BAC vector, pBeloBac11, uses a complementation of the lacZ gene to distinguish insert-containing recombinant molecules from colonies carrying the BAC vector, by color. Kim, U-J et al. Thus, it is now possible to distinguish those colonies that contain BACs with inserts from those that lack inserts. Although these single-copy vectors are advantageously used to clone large genomic DNA fragments for subsequent analysis, especially sequence analysis, the single-copy nature of these vectors is also a limitation in that large numbers of cells containing a BAC clone of interest must be grown to produce a sufficient quantity for subsequent analysis. It is, of course, possible to amplify portions of a BAC clone of interest using, a for example, PCR, but simple amplification of the entire insert from a BAC vector has not previously been possible. In , Szybalski proposed a system for fragment-by-fragment sequencing of entire bacterial genomes by in vivo excision and subsequent amplification of the excised genomic fragments. One aspect of the Szybalski system is that the excision from the bacterial chromosome and the amplification of the excised genomic fragment be controlled very stringently so that excision is induced only on command and amplification is initiated only upon induction. According to the proposed system, excision-mediating sites EMS are placed at kb intervals throughout a well mapped genome in places where the EMS do not interfere with viability or genomic stability. The EMS are placed throughout the system either by 1 randomly inserting plasmids carrying transposons, EMS, and selective markers, or 2 by targeting insertions that inactivate specific, non-essential genes. The net result of adding EMS to a genome in the Szybalski system is a library of strains, each of which carries one EMS at a physically mapped site. Using genetic crosses between strains having neighboring EMS at a suitable distance from one another, a set of strains are produced where each strain possesses exactly two neighboring EMS. From such strains, the intervening segment e. Accordingly to the Szybalski system, if a cis-acting ori element is positioned together with and next to the excision elements, the ori element will be present on the excised DNA circle and can promote the amplification of the DNA circle. Szybalski does not contemplate employing an amplification and excision system in conjunction with a bacterial artificial chromosome. Such a system would provide additional benefit in that it would not require the steps of interspersing EMS throughout the genome and crossing EMS-containing strains, but could instead rely upon the use of rare-cutting restriction enzymes to generate genomic fragments of suitable size for cloning into a BAC vector. In the presence of a suitable signal in a suitable host cell, the EMS of an insert-containing BAC clone are conditionally activated to recombine with one another and thereby excise the nucleic acid therebetween to produce a circular plasmid that comprises the genomic fragment insert. Both the EMS and origin of replication ori are responsive to trans-acting signals that direct the fragment to be excised from the vector and to be amplified by replication. The conditional ori is positioned such that it resides on the plasmid excised from the BAC clone in the excision step. In response to a suitable signal in the host cell, replication is initiated at the ori and the excised plasmid is amplified and accumulates in the cell at high-copy number. Any BAC vector can be improved in accordance with the present invention. Before being improved, the BAC vector must be capable of independent replication in the host cells. The vector should therefore include an ori that functions in the host cells, as well as any other genes that encode proteins required for plasmid replication, maintenance, and partitioning. The vector can contain a selectable marker such as a chloramphenicol-resistance gene, and at least one cloning site into which the genomic fragments can be cloned. The vector also preferably contains a

gene that can be disrupted by inserting a genomic fragment into the cloning site, thereby imparting an ability to distinguish clones that contain a genomic insert from those that lack a genomic insert, preferably on the basis of color. Modifications in accordance with the present invention are described relative to pBeloBac11, although one skilled in the art can readily make the same changes to other plasmids including, but not limited to, pBacL. In a second aspect, the invention is a host cell comprising in its interior the improved BAC vector of the present invention, where the host cell can conditionally provide the signals required to excise and amplify the insert from the BAC vector. The host cell is preferably bacterial, such as E. In a third aspect, the invention is a method for amplifying in a host cell a genomic DNA insert in a BAC vector modified in accordance with the invention. The method includes the steps of 1 providing in the host cell a first signal that directs excision of the genomic DNA insert at the EMSs to form an first plasmid that comprises the excised insert and an ori competent in the host cell, and a second plasmid that comprises the BAC vector backbone, and 2 providing in the host cell a second signal that directs amplification of the excised plasmid to replicate, thereby producing multiple copies of itself. Existing pBAC vectors could alternatively also be improved simply by providing a conditional ori on the vector. Such a modified vector with insert would be maintained at a single copy, but could be amplified in its entirety upon command. Such a vector could be prepared as described herein, except there would be no need to provide the EMS or the excision-mediating protein. A conditional activator of the ori would still be required, and could be provided as described herein. It is an object of the present invention to retain the advantageous properties of existing BAC vectors. It is another object of the present invention to provide a bacterial artificial chromosome that can, in the presence of an appropriate first signal, excise a sequence of interest from the BAC which can, in the presence of a second signal, amplify the insert to a copy number of between 10 and copies or higher. It is yet another object of the present invention to provide a vector that can be conditionally amplified in the presence of an appropriate signal. It is a feature of the present invention that the BAC vector includes a pair of excision mediating sites EMS flanking a site into which genomic DNA fragments can be cloned and furthermore contain a conditional ori near one EMS and in any event between the pair of EMS that flank the cloned gene. It is an advantage of the present invention that an abundance of a target insert in a BAC can be selectively replicated by providing appropriate signals to the host cell. Other objects, advantages, and features of the present invention will become apparent upon consideration of the following detailed description taken in conjunction with the accompanying drawings. The vector also includes a lacZ gene that can be disrupted or eliminated from the vector when an insert is cloned. It is not essential that the EMS and the ori be immediately adjacent to one another, although this can be convenient in the laboratory. It is essential, however, that the ori element is more proximal than the FRT element to the MCS so that the conditional ori element is provided on the same segment of the BAC vector as the cloning site. This short sequence is the target for the yeast Flp protein. The preferred conditional ori is oriV, although the conditional ori could be any ori that functions in the host cell and is responsive to the amplification-mediating protein s. It is preferable, for the sake of simplicity that the ori respond to a single protein, although this is not essential, and multi-protein replication systems are known. It is preferable that the ori amplify the plasmid to high copy number where the goal is to produce large quantities of DNA. The oriV is preferred because of its broad host range, its known capacity to replicate DNA fragments of kb or larger, its high copy number and its requirement for only one inducing protein. The orientation of the two joined fragments is such that when the fragment is cloned into the XhoI site, the ori is physically located between the nearby FRT site and the insert cloning site. It is important that the EMS be inserted in parallel orientation to the EMS inserted to the opposite side of the cloning site. Since excision will occur only if the two EMS are in parallel orientation, one can readily determine whether the proper insertion has been made by performing the excision assay noted below. If excision is not noted, then the second EMS is not in parallel with the first and is not appropriate for further use according to the invention. The resulting improved vector, depicted schematically in FIG. Insert-containing clones of interest can be obtained for further analysis from the resulting libraries using the same techniques as are described in Kim, U-J. An

advantage of the present invention is that in addition to its capacity to maintaining and yielding only a single copy of the cloned fragment, multiple copies can be easily produced without requiring additional cloning steps. Once an insert-containing clone of interest is identified, the conditional excision and amplification of the present invention can be induced, but only on command. To excise the fragment of interest, the cells containing the BAC vector are induced to produce the excision-mediating protein. In the presence of Flp, which interacts with the pair of parallel FRT sites, the fragment of interest is excised and circularized. Since the excised insert-containing plasmid contains the conditional ori, in the presence of the amplification-mediating replication protein s the plasmid is induced to replicate from the conditional ori until it reaches its maximal copy number, which can typically range between 10 and In the preferred embodiment, employing the oriV, the plasmid is induced to replicate to high copy number in the presence of TrfA protein. The sequence of TrfA is known as is the sequence of the gene that encodes TrfA. In the preferred embodiment, copy-up mutant trfAD was employed, although others could be used. Several suitable means exist for providing the excision-mediating and amplification-mediating functions in the host cell that contains the modified BAC with insert. Since the excision and amplification are only desired at a certain circumstance or stage in the process, the genes that encode the required proteins are under tight regulatory control, without regard to the means by which the functions are provided. In the exemplified embodiment, the FLP and trfA genes were provided adjacent to one another in a cassette under the transcriptional control of the Ptet promoter which can itself be repressed by the TetR protein. The tetr gene was provided, to ensure the presence of TetR. When the TetR repressor activity is eliminated, in the presence of autoclaved chlortetracycline, the genes that encode the excision- and amplification-mediating proteins are transcribed and translated, whereupon excision and amplification of the cloned fragment can proceed. A first means for providing the controlled activation requires the host cell chromosome to include the genes that encode both functions, and this method is considered preferred by the inventor, since it does not require the maintenance of a delivery plasmid. To insert the above-noted cassette into the host genome, the cassette can simply be provided on a plasmid that also includes an attP integration site, a temperature-sensitive ori, and an antibiotic-resistance-encoding gene such as a gene encoding kanamycin resistance. The plasmid can be transformed into a host cell such as E. The unintegrated plasmid will not persist at the elevated temperature, since it can only replicate at the permissive temperature, and thus it will become diluted. If the genes that activate the excision and amplification functions are not provided in the host cell chromosome, a comparable regulated cassette can be provided on a low-, medium-, or high-copy number plasmid that resides within the host cell. The cassette can readily be ligated into a backbone of, for example, low-copy-number plasmid RSF, medium-copy-number plasmid pBR, or high-copy-number plasmid pBBR1 in a manner known to one skilled in the art, where the backbone provides the plasmid replication functions. The plasmids can be introduced into a host cell, typically an E. In addition to varying copy numbers, the delivery plasmid that supplies the trans-activating functions can be chosen on the basis of host range. Procedures for preparing such plasmids and for delivering the plasmids into host cells are described in Wild, J. In the method of the present invention, BAC libraries are prepared using the vector of the present invention using the same techniques as are now presently employed using e. Briefly, size selected high-molecular-weight DNA is partially or completely digested with a rare-cutting restriction endonuclease such as NotI, and the size-selected DNA is ligated into the vector of the present invention at an approximately Library screening is accomplished as previously described in the above-incorporated papers. When a clone of interest is identified for further analysis, the cells of the clone are grown overnight in LB medium supplemented with appropriate antibiotics to ensure that the cell maintains both the BAC clone as well as the delivery plasmid if required. The overnight cultures are used to inoculate fresh M9 minimal medium Sambrook, J. During this incubation, the fragment between the FRT sites that contains the cloning site and the conditional ori is excised from the BAC clone and amplified to the levels described. DNA is extracted from the overnight cultures by alkaline lysis. Although it is preferred that the resulting DNA be somewhat purified before further analysis, especially if the DNA will be fragmented for use in shotgun cloning, purification is not

considered an essential step of the process. Purification may not be required for subsequent use of the DNA for sequence analysis by primer walking. The present invention will be better understood upon consideration of the following non-limiting example. The cells were either not induced control or induced with autoclaved chlortetracycline cTc. DNA was obtained from the control and induced host cells as described herein, and was cleaved with NcoI. NcoI-digested uninduced vector produced only two fragments, long and short, which can be resolved visually from one another after electrophoresis. Notably, the band that corresponds to the fragment that contains the oriV and the cloning site is wider and brighter than any other band, even despite its smaller size. This is indicative of targeted amplification of that one oriV-containing and excised plasmid, as would be expected. Thus it is shown that a system for excising and amplifying an insert from a BAC clone in a tightly controlled manner is an advantageous improvement over existing pBAC systems and a vector that includes excision mediating sites flanking both a site into which a genomic clone can be inserted and a tightly controlled ori, is also an improvement over existing pBAC vectors. A bacterial artificial chromosome comprising a site into which an DNA fragment can be cloned; a pair of inducible excision-mediating sites flanking the site into which the DNA fragment can be cloned, the excision-mediating sites being provided in parallel orientation relative to one another and defining an excisable fragment that comprises the site into which the DNA fragment can be cloned; and an inducible origin of replication on the excisable fragment. A bacterial artificial chromosome as claimed in claim 1 wherein the pair of excision-mediating sites are FRT sites. A bacterial artificial chromosome as claimed in claim 1 wherein the pair of excision-mediating sites comprise a sequence as shown in SEQ ID NO: A bacterial artificial chromosome as claimed in claim 1 wherein the inducible origin of replication is oriV. A cell comprising in its interior a bacterial artificial chromosome that comprises a site into which an DNA fragment can be cloned, a pair of inducible excision-mediating sites flanking the site into which the DNA fragment can be cloned, the excision-mediating sites being provided in parallel orientation relative to one another and defining an excisable fragment that comprises the site into which the DNA fragment can be cloned, and an inducible origin of replication on the excisable fragment; a gene encoding an excision-mediating protein that induces the excisable fragment to excise from the bacterial artificial chromosome and circularize to form an excised plasmid; and a gene encoding an amplification-mediating protein that induces the excised plasmid to replicate. A cell as claimed in claim 5 wherein the gene encoding the excision-mediating protein is a FLP gene.

4: Publications Authored by Peter J Murray | PubFacts

An approach is described to modify yeast artificial chromosomes (YACs) with cassettes that can be easily excised for embryonic stem (ES) cell gene targeting experiments.

5: YAC Protocols - CiteSeerX - www.enganchecubano.com

Targeting Vector Construction by Yeast Artificial Chromosome Modification. Peter J. Murray. the wide range of uses for large insert yeast artificial chromosome.

6: Yac Protocols - Mackenzie Alasdair (Curatore) | Libro Humana Press 08/ - www.enganchecubano.com

[et al.] -- Genomic reconstruction by serial mitotic recombination of yeast artificial chromosomes / David Markie, Emma Jones, and Jiannis Ragoussis -- Targeting vector construction by yeast artificial chromosome modification / Peter J. Murray --, Production of yeast artificial chromosomes transgenic mice by pronuclear injection of one-cell.

7: YAC Protocols â€“ Buchhandlung Buchkultur

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It is now 10 years since the first edition of YAC Protocols was published in YAC Protocols was first produced to address the huge demand within the research community for a lab-based text that described in detail the wide range of uses for large insert yeast artificial chromosome (YAC) DNA clones.

8: YAC Protocols 2nd Ed (Methods in Molecular Biology Vol) - PDF Free Download

The completed targeting vector can be transfected into C57BL/6 ES cells and clones selected with G followed by injection into Balb/c blastocysts. YTT increases the speed of targeting vector construction and obviates the need for extensive backcrossing to the C57BL/6 background.

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