Exo-LMPCR surpasses existing methods due to combined properties of being universal and directional without reliance on restriction sites, streamlined for superior flexibility in control of 3' and 5' end adaptors, and extremely robust due to specific assembly and efficient ligation.

2286

A Novel Cre-Lox System for Rapid Construction of First Generation Adenovirus Vectors

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Adenoviral (Ad) vectors are advantageous for gene delivery because of their high titer and ability to efficiently transduce a wide variety of cell types *in vitro* and *in vivo*. However, early generation Ad vectors, devoid of E1 sequences (and often E3 sequences), have not been widely used partly because of the difficulty in cloning into the large Ad backbone (~30kb). Traditionally, cloning into Ad vectors was done by homologous recombination in human cells. However, this is inefficient and requires screening of multiple plaques. Recombination in bacteria has also been used, but is complicated by the need to transfer the vector through multiple strains to yield a usable construct. A direct ligation strategy using "rare-cutter' restriction enzymes works well, but still requires several steps to purify the digested fragments. Here, we present a simplified method to produce recombinant Ad constructs using *in-vitro* cre-lox recombination (the Creator System) to directionally and precisely introduce a gene of interest into an Ad backbone. Using a Creator-compatible Ad backbone and multiple cDNAs, we achieved >95% cloning success with little or no background as measured by colony PCR screening and DNA digestion and analysis. To determine functionality, the pLP-AdLacZ vector was tested by virus rescue in 293 cells and subsequent titration. Expression from the LP-AdLacZ vector was also determined by infection of HeLa cells and quantitative measurement of LacZ expression by luminescence detection. Our data demonstrates this virus construct could be grown to high titers (>1e11ifu) and that additional cloning elements within the backbone did not affect virus functionality or expression. We believe the Creator-based Ad cloning system will increase the utility of adenoviral vectors by allowing rapid, accurate, and directional cloning, thereby facilitating production of functional adenoviral stocks.

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Replication of Bacterial PlasmidsContaining Map Units88-100 of Parvovirus LuIII in EukaryoticCells

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The synthesis of plus strand by parvovirus LuIII requires replication from the 5' terminus of the minus strand DNA. An A/T rich region identified in LuIII has been suggested to represent a possible origin of replication at the 5' terminus of the virus. In an attempt to identify this origin the replication of two clones was studied. Map units 88-100 of LuIII were cloned (clone pUraLu88-100) into pGN3, a pUC derived plasmid, containing the URA3 gene of *S.cerevisiae* as an auxotrophic marker. pUra-Lu88-100 was transformed into *S.cerevisiae* (SEY S288C,ura3-) and plasmid DNA was subsequently isolated from the resulting colonies and transformed into *E.coli* DH5α cells. Restriction enzyme, Southern Blot and sequencing analyses of the plasmid rescued from yeast confirmed the presence of LuIII and URA3 sequences. pUra-Lu88-100 was capable of existing in a free state in *S.cerevisiae*, suggesting that the elements required for autonomous replication were present in the plasmid. In an attempt to confirm this ARS-like function observed in yeast in higher eukaryotics, the URA3 gene was removed from pUra-Lu88-100. This new clone, pLu88-100 was used to transfect Human Embryonic Kidney Cells (NBE 324K) . pLu88-100 resistant to *DpnI* digestion was recovered from 324K cells, confirming that as observed in yeast the plasmid was capable of autonomous replication.

2288

Development of a plasmid vector for sustained gene expression *in vivo*

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Delivery of therapeutic proteins by administration of the genes that encode these proteins has significant clinical potential. However, a major hurdle of naked DNA gene therapy is that protein expression is transient. Using the viral cytomegalovirus (CMV) promoter to drive the expression of the transgene does result in high protein expression, but the expression is short-lived. We tested several non-viral promoters and found that they were less effective than the CMV promoter and failed to produce long-term expression. Since mammalian chromosomes are not circular, we wanted to test the effects of linearization of the plasmid. We changed the plasmid structure by cutting once with a restriction enzyme to linearize it. We also tested the removal of the vector backbone by cutting the DNA at two restriction enzyme sites. We fragmented and linearized the constructs with either the CMV promoter, or a tissue-specific promoter. The reporter gene human secreted alkaline phosphatase (hSEAP) was used in these vectors for our experiments, and plasma was assayed for levels of alkaline phosphatase. In vivo experiments demonstrated that excision of the bacterial backbone in conjunction with a tissue specific promoter resulted in sustained protein expression for over 2 months. In contrast, protein expression levels were not detectable at this time point with circular plasmid DNA. We conclude that the plasmid structure and the promoter are important factors to consider when designing a plasmid for gene therapy.

2289

Controlledefficacy transfection of myoblasts with lipid-based reagents

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Background: A potentially powerful approach in the treatment of disease involves using cells to introduce genes encoding therapeutic proteins into the body. Myoblasts are a potent tool for stable delivery of genes, as they are relatively ischemia resistant and become an integral part of muscle into which they are injected. Recent development of improved lipid-based reagents potentially allows for fine control of recombinant gene expression levels in myoblasts, which could provide a powerful toolbox with which to develop gene therapies for a number of human diseases. To define conditions for a range of gene expression, we compared commercially available lipid-based and polyamine transfection reagents for reproducible gene delivery into myoblasts.**Methods:** the mouse C2C12 cell line was transfected with one of two reporter genes or a reporter gene co-expressing Nkx2.5 (pCMSEGFP: 5.5kb, pDsRed2C1: 4.7kb and pCMSEGFP/Nkx2.5: 6.8kb). Seven reagents were screened using optimal conditions suggested by the manufacturers. In optimization experiments, 1.0, 2.0, 4.0 ug of DNA were used per 3xE4 cells. Two microgram of DNA was then utilized to identify the optimal DNA (ug) / reagent (uL) ratio.**Results:** Efficacies ranged from 2.5% to 32% with pCMSEGFP (Table). The highest average of efficacy could be obtained with the combination of 2ug of DNA and 6uL of FuGENE6.**Conclusions:** Both the quantity of DNA and the ratio of DNA:Reagents had a significant effect on transfection. The degree of gene delivery into myoblasts can be manipulated by varying the amount of DNA, the ratio of DNA:Reagents and the reagent used. This potentially allows for control of DNA levels in cells and thus for use in gene transfer in disease states. This kind of control can be obtained without the need for viral vectors.

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A Novel Two-Step Recombinogenic Engineering Method for Bacterial Artificial Chromosome Manipulation

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Homologous recombinogenic engineering is a useful method to engineer DNA without the need for restriction enzymes or ligases using a set of lambdaphage proteins. Construction of a Tyrp-1 (Tyrosin related protein 1, Tyrp-1) BAC containing upstream regulatory elements has been a significant challenge as library derived BAC have been split into two BAC's, one containing the regulatory elements, and the other containing the exons. In this work, a full-length Tyrp-1 BAC is constructed from the original two library-derived BAC's using a novel two-step method based on the lambda phage Red proteins. Such recombination of BAC's represents a new application of homologous recombination, and will be a powerful new tool in BAC DNA engineering.

2291

Transcriptional Activation of the Endogenous VEGF Gene by Small Molecule Regulated Engineered Transcription Factors

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Engineered transcription factors based on the C2H2 zinc finger DNA binding motif have the capacity to recognize any cellular gene. When linked to a functional domain (for example; a transcriptional activation or repression domain) these proteins function as potent and specific regulators of gene activity.We have designed proteins that are transcriptional activators of the endogenous VEGF gene (Liu et al. (2001) J. Biol. Chem. 276:11323-11334). These activators are currently being evaluated as stimulators of therapeutic angiogenesis for the gene therapy treatment of ischemic heart and limb diseases. We would like to be able to regulate the activity of our transcription factor proteins to allow more precise control over expression of the VEGF gene.Several technologies are available for the inducible switching of gene expression. We chose to use the Valentis GeneSwitch® system, originally developed by Bert O'Malley (Wang et al. (1994) PNAS 91:8180-8184), as this gave us the option of directly modifying our proteins to make them regulatable. We fused the C2H2 zinc finger domain from our VEGF regulating protein to the progesterone receptor ligand binding domain and p65 transcriptional activation domains from the GeneSwitch® regulator protein. In the absence of drug this protein is rendered inactive by sequestration in the cytoplasm, but on addition of mifepristone it is translocated to the nucleus, where it is transcriptionally active. We have shown that this construct exhibits tight mifepristone-dependant regulation of the endogenous VEGF gene in a dose dependant manner in human and murine cell types. An in-vivo evaluation of these constructs is currently underway.This capacity for small molecule control of endogenous gene expression will be potentially beneficial in both experimental and therapeutic applications.