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Laboratory of Comparative Genomics

Recombineering: Development of BAC-based Experimental Analysis

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Abstract

Bacterial artificial chromosomes (BACs) provide valuable resources for the analysis and manipulation of large genomic fragments. Using recombineering to make directed genetic modifications of genomic DNA cloned into BACs, we are developing approaches to assist in the resolution of complex human diseases with the pig biomedical model. To overcome difficulties associated with the introduction of BACs with large inserts into mammalian cells, we carried out experiments to determine the effects of various parameters regarding the efficiency of DNA transfection into porcine fibroblast cells. The BAC clone 403O9 from the RPCI-44 library was recombineered by introduction of a cassette encoding enhanced green fluorescent protein (EGFP) and used to monitor the effectiveness of BAC DNA uptake into the fibroblast cells. Since the rate of successful BAC transfection depends on factors including the purity of BAC DNA, the transfecting agent, and the target cell line, we compared the effects of varying these parameters on transfection efficiency. The efficiencies were determined by flow cytometry analysis of transient EGFP expression in the transfected cells. We determined that the time course of EGFP expression varied depending on method of transfection (non-liposomal lipid reagent with DNA-condensing enhancer, lipid-based transfection reagent, or electroporation), observed the transfected cells expressed EGFP for up to two weeks, and optimized the conditions for transfection. Co-transfecting an EGFP BAC with another BAC of interest may provide a rapid method of selecting successful transfectants.

Introduction

- Recombineering provides a rapid method to genetically modify genomic DNA inserts cloned into BACs. In our laboratory we have targeted genes that will be useful to create biomedical research models. Using the recombinogenic *E. coli* strain DY380, we have generated multiple genetic modifications in BAC clones.
- Another component in developing a recombineering platform is the ability to use the recombineered BACs to introduce targeted changes in fibroblast and other somatic cells. Introduction of a mutation into a mammalian cell by homologous recombination requires that the region of homology of the construct and targeting sequence be as large as possible, however, the large size of the BAC insert makes it difficult to efficiently transfect the cells.
- Here we have utilized the porcine BAC 403O9 that contains the myostatin gene to develop a system to monitor the transfection efficiency by transient expression of the EGFP from the recombined BAC.
- Lipid based delivery systems have proven extremely effective for achieving high rates of plasmid transfection in mammalian cells. Here we compared the efficiency of two different delivery systems, Effectene and Lipofectamine 2000.

Objectives

- Develop a system to test transfection efficiency of BAC into porcine fetal fibroblast cells.
- Determine the optimal method for transfection and time of EGFP expression after transfection.
- Improve the method by varying the parameters to increase the efficiency of BAC transfection such as quantity of DNA, amount of transfecting agent used, cell line, and finally purity of DNA.

Materials and Methods

❑ **Construction of targeting vector for EGFP insertion:**

Construct was amplified from pEGFP –N1 vector DNA using 90bp primers. Each of them had 70bp homology with the region of the myostatin gene upstream and downstream of the insertion site.

*Dpn*I cleaves methylated GATC DNA and digests pEGFP-N1 DNA , preventing contamination with EGFP-expression vector

❑ **Extraction of BAC DNA**

NucleoBond BAC Maxi Kit (BD Biosciences) (NB)
Reliable, high yield
60ug/100 mls

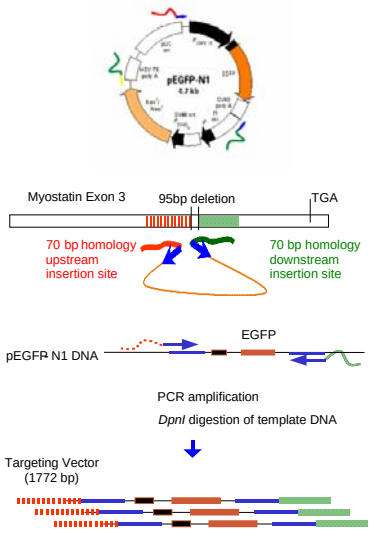
EndoFree Plasmid Maxi Kit (EndoFree)
High efficiency for transfection of mammalian cells
High purity DNA, close to CsCl extracted DNA
10ug/100 mls

❑ **Cell Transfection**

UIUC Y12F cell line was transfected with either BAC DNA or plasmid DNA

❑ **Expression Analysis**

EGFP gene expression monitored on days 4, 7, 10, and 14 by flow analysis of 50,000-100,000 transfected porcine fetal fibroblast cells



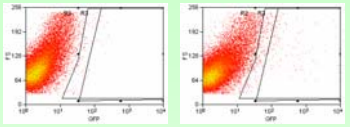
Transfection Conditions

Transfection Method		Culture Dish	Confluence	Media Type	DNA (ug)	Reagent (ul)	37° Incubation (hours)
Lipofectamine 2000	Invitrogen	60 cm²	60% UIUC Y12F	Opti-MEM Reduced Serum Medium	8 ug	8 ul Lipofectamine	5 hours
Effectene Transfection Reagent	QIAGEN	60 cm²	60% UIUC Y12F	15 % D-MEM	2 ug	16 ul Enhancer 40ul Effectene	24 hours

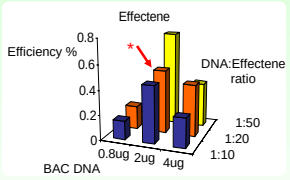
Results

Effectene

Control | NB extracted DNA (170 kb)



Region	Count	%
R2	605	0.57%
R3	192	0.20%

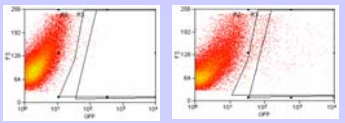


* Optimal condition

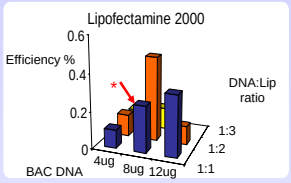
Effectene Transfection Reagent is a non-liposomal lipid formulation in which the Enhancer condenses the DNA molecules and Effectene subsequently coats them with cationic lipids.

Lipofectamine 2000

Control | NB extracted DNA (170 kb)

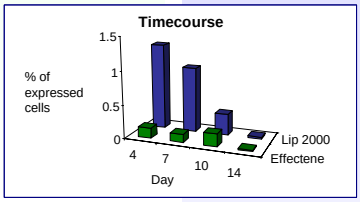


Region	Count	%
R2	923	1.01%
R3	435	0.52%

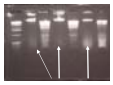


* Optimal condition

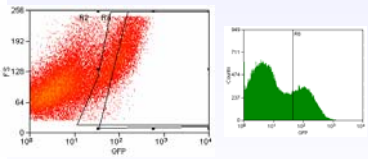
Lipofectamine 2000 is a catioinc lipid-based transfection reagent optimized to work for many mammalian cell lines.



EndoFree extracted BAC DNA (170kb)

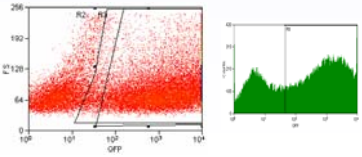


Region	Count	%
R2	15878	22.87%
R3	8415	12.37%



EndoFree pEGFP-N1 plasmid (4.7 kb)

Region	Count	%
R2	34527	72.77%
R3	32343	68.24%



Conclusions

- We developed a useful system to check for transfection efficiency through the expression of EGFP by fetal fibroblasts as measured by flow cytometry.
- This study showed the optimal method for transfection of our BAC DNA into UIUC Y12 fetal fibroblast cells was achieved using the EndoFree kit for DNA extraction and Lipofectamine 2000 for transfection. The efficiency of transient expression of recombineered myostatin BAC (170 kb) was approximately 22.87% using these parameters. However, the results were inconsistent for different experiments.
- The transfection efficiency of vectors with large genomic inserts greatly depends on the insert's size. To overcome the low efficiency and inconsistency with transfection of 150-200 kb inserts we are currently constricting the series of vectors with smaller inserts of 15-25 kb. The same recombination system is used to subclone fragments from original BAC into pBR322 by gap repair.

Acknowledgements

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