Construction of a Lentiviral Vector Expressing EGFP-tagged human CFTR for Testing Gene Transfer in Cystic Fibrosis Animal Models

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Abstract

Cystic fibrosis (CF) is a genetic disorder, caused by mutations in the CFTR gene that encodes CFTR, a chloride channel. CF affects 1/~3000 live births in the United States. Clinically, adult patients suffer mostly from lung complications, characterized by chronic bacterial infection, persistent neutrophilic inflammation and mucopurulent airway obstruction. While treatments to improve the quality of life and lifespan of afflicted people are practiced, there is no cure for this disorder. As CF is caused by gene mutations, gene therapy presents a potentially wide-spread and life-long treatment. To test this concept, we aimed to construct a new lentiviral vector designed to express eGFP-tagged human CFTR, which will allow us to trace and evaluate gene transfer. First, human CFTR (hCFTR) cDNA was amplified via PCR from a carrying plasmid with a set of primers that have tail sequences homologous to a lentiviral vector plasmid that expresses eGFP driven by the EF1α promoter. The hCFTR amplicon is annealed to the lentiviral vector plasmid and extended via PCR so that hCFTR gene is fused in frame with the eGFP gene at its C-terminus. After a heat-shock transformation of E. coli competent cells, overnight culture gave rise to hundreds of colonies, which were screened via phenol extraction and gel electrophoresis. The colonies of the correct size were obtained. Currently, we are doing plasmid preparation and sequencing to verify the correctness of the expected eGFP-hCFTR lentiviral plasmid. Once it is confirmed, a large preparation of the plasmid will be produced and used for transfection of a new and permanent lentiviral producing cell line to produce the needed lentiviral vector for CFTR gene transfer to CF cell lines and CF animals.

Workflow

1. PCR amplification of human CFTR gene (hCFTR)
2. Fusion of hCFTR gene with EGFP gene in a Lenti-EGFP plasmid via PCR amplification
3. Transformation of the amplicon into competent E. coli cells.
4. Overnight culture of the transformant on Kanamycin agar plates
5. Colony isolation and expansion by streaking on new Kanamycin plates
6. Colony screening using phenol extraction from toothpick-collected bacterial samples.
7. Gel electrophoresis to identify the potential colonies.
8. Overnight LB-culture of the potential colonies
9. Plasmid isolation via Qiagen mini prep method for sequencing

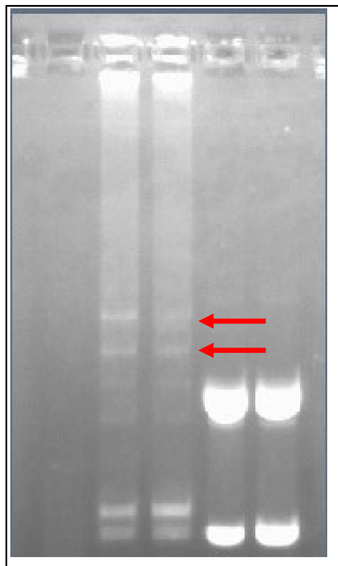
Methods

PCR conditions: For hCFTR amplification, 98 °C 30'', 98 °C 10'', 65 °C 30'', 72 °C 160'' for 29 cycles. For fusion of hCFTR and EGFP in the lentivector, 98 °C 30'', 98 °C 10'', 65 °C 30'', 72 °C 390'' for 29 cycles.

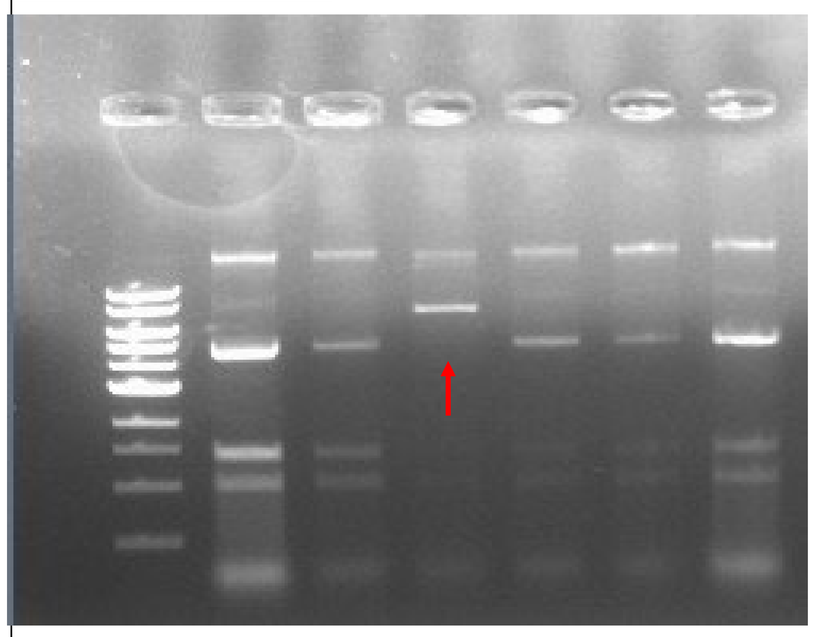
Transformation: Competent E.coli cells were allowed to dethaw on ice before being cultured in liquid media and concentrated. After concentration, the E.coli was mixed with the plasmid created earlier and heat shocked at 42 °C for 45 seconds. The tubes were then rested for another 30 minutes on ice before being allowed to create colonies overnight for harvesting and later plasmid testing.

Bacterial Screening: 1.5 mL microcentrifuge tubes were numbered for each colony tested and had 35 uL of TE buffer added to each. 7 uL 6x loading buffer dye were added to each tube, diluted to reach 1x. Toothpicks were used to scrape up colony matter based on number and add to the TE buffer + loading dye solution. 42 uL Phenol were added to each now differentiated tube, tubes were vortexed and centrifuged for 10 minutes. Each sample was loaded into a 0.8% agarose gel and run on 100 V.

Results



Fusion of the EGFP and hCFTR genes in a Lentiviral vector plasmid via PCR amplification. Red arrows indicate the amplified plasmid the gene fusion.



A representative gel image showing colony screening. Red arrow indicates a correct colony with the expected plasmid size.

Conclusion

A new lentiviral vector expressing an EGFP-hCFTR fusion protein has been constructed. The correct bacterial clones have been identified and are currently under further characterization.

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