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Modified bacterial method for the generation of recombinant adenoviruses via super-heat-shock

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Article Outline

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Recombinant adenoviruses are routinely used to study gene expression and also for therapeutic purposes (Ref. [1-3]) such as gene delivery. Most investigators generate the recombinant adenoviruses (with their gene of interest incorporated) via homologous recombination in *Escherich coli* rather than by recombination in eukaryotic cells, because the latter method is less efficient. Unfortunately, even the bacterial method has so far proved to be inefficient and not reproducible. Thus, it is necessary to have a reproducible, simple process to generate recombinant adenoviruses.Here, we describe an improvement of the bacterial method. Our method is based on the standard AdEasy system (http://www.coloncancer.org/), using heat shock rather than electroporation for the co-transformation of the DNAs (for homologous recombination) into the BJ5183 bacterial cells.

Protocol to generate the pertinent recombinant adenovirus

The gene of interest is first cloned into the multiple cloning site of the pAdTrack-CMV vector by standard methods. The transformation is done in DH5α bacterial cells.

A 20 ml culture in LB (with 50 μg kanamycin per ml) of this transformed plasmid is grown overnigl and the plasmid isolated by means of the RPM Turbo Kit (Cat. No. 2066-200, Q-BIOgene, Carlsbac CA, USA).

This plasmid is then linearized by digestion with the restriction endonuclease *Pme*I and run on a 1' agarose gel (with ethidium bromide in the gel at a final concentration of 0.2 μg ml^{-1}) in TBE for an appropriate period until the relevant band of this linearized plasmid is observed to be clearly separated. This band is then cut out and the DNA isolated by means of a RPM Turbo Cartridge (not suggested use by the manufacturer). The excised band is dissolved in RPM Turbo Salt Solution (approximately a threefold excess of this solution by weight in relation to the gel slab) and heated 62 °C until the gel dissolves and then cooled to room temperature. This is then adsorbed onto a RI

Turbo Cartridge and the protocol suggested by the manufacturer followed to isolate the linearized plasmid DNA from this cartridge.

BJ5183 bacterial cells (from a single colony from a LB–agar–streptomycin-sulfate plate or from a liquid culture stored at -20 °C in glycerol) are grown overnight in LB with 50 μg streptomycin sulfa per ml at 37 °C. Both these sources gave similar transformation efficiencies. To 20 ml of LB (with μg streptomycin sulfate per ml) is added 100 μl of this overnight culture of BJ5183 cells and this is grown until absorbance at 550 nm is 0.8–1.0.

These cells are then spun on a centrifuge at 2244 *g* for 10 min at 4 °C and the supernatant decant Following this, 20 ml of ice-cold calcium chloride (0.1 M) are added to these cells and the cells gen vortexed and placed on ice for 30 min. These cells are again spun at 2244 *g* for 10 min at 4 °C. Following this, the calcium chloride solution is decanted and 0.5 ml of cold calcium chloride (0.1 M added to these cells and they are then resuspended by gentle vortexing.

1.0–1.5 μg (in 5–10 μl sterile distilled water) of the *Pme*I-linearized pAdTrack-CMV vector (with th gene of interest) is co-transformed with 400 ng (in 5–10 μl sterile distilled water) of adenoviral backbone (pAdEasy-1) in 100 μl BJ5183 cells from the treatment above. This mixture is then gentl vortexed and placed on ice for 1 h. Subsequently, this mixture (in 1.5 ml Eppendorf tubes) is heat shocked in a water bath for 5 min at 45 °C. After this, the mixture is placed on ice for 5 min and 5 μl LB medium added. The mixture is next transferred into 15 ml Corning tubes and grown in a sha for 1 h at 37 °C. Next, 300 μl of this mixture are plated on each LB–agar–kanamycin (25 μg kanamycin per ml) plate and grown for 24–36 h.

10–15 tiny colonies are isolated from these plates and then grown in 20 ml LB–kanamycin (25 μg kanamycin per ml) and the plasmids isolated next day using the RPM Turbo kit. These plasmids ar digested with *Pac*1 and run on a 1% agarose gel (with ethidium bromide) in TBE. The encouraging candidates are transfected into the 293 cells by means of the Polyfect reagent (Cat. No. 301105, Qiagen, Valencia, CA, USA) following the manufacturers protocol. The transfection was followed by the production of green fluorescent protein. Following cell lysis, the virus with the pertinent gene i isolated.

Although most investigators (e.g. Ref. [4]) tend to use electroporation for the necessary co-transformation of the DNAs, this procedure requires a specific apparatus coupled with fine tuning. Moreover, in order to obtain some degree of reproducibility by this method, it is necessary to purif the adenovirus backbone (AdEasy) by CsCl banding (http://www.coloncancer.org/). In our methoc no special apparatus or treatment of the DNA are necessary. In addition, the proportion of homologous recombinants is higher (80–100%; Table 1) than those by the traditional electroporat method (Ref. 4). Also, the yields of the precise recombinants can often be above 50% (Fig. 1) of total number of colonies that were screened. In fact, using this approach, we have routinely obtair as many as 100% precise recombinants.

Table 1. Comparison of recombinants (candidate and precise) generated by electroporation and super-heat-shock

Electroporation	Super-heat-shock
~100 colonies were obtained (Ref. 4)	~80 colonies are typically obtained
Of these, ~67% were candidate recombinants	Of these, 80–100% were candidate recombinants.
No mention was made the proportion of precise recombinants	50–100% of the resistant colonies screened were usu precise recombinants.
	It was often sufficient to examine just 15 colonies to obtain the precise recombinants.

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Fig. 1.
Vascular-endothelial-growth-factor adenovirus recombinants generated by this method. Lanes 1 and 19 are phage-λ-DNA *Hind*III-digest markers. All other samples are *Pac*I restriction-enzyme digests. Lane 2 is the pAdTrack-CMV with VEGF cloned in it (negative control), whereas lane 18 is the recombinant adenovirus with no insert (positive control). Except for lanes 4, 11 and 12, all other samples are recombinants. Lanes 5, 7–10 and 14–16 represent the appropriate VEGF adenovirus recombinants. The proper clones are known to yield a large fragment (near 30 kb) an a small fragment of 3 kb or 4.5 kb with *Pac*I digestion.

We observed that heat shock at 45 °C for 5 min gave optimal results. The traditional method of he shock at 42 °C for 90 sec (Ref. [5]) gave poor co-transformation and no desired recombinants.

Finally, it is still not clear to us precisely why we obtain a higher proportion of recombinants using

this approach. A plausible explanation is that, owing to super-heat-shock, the co-transformation or retention (or a combination of transformation and retention) of DNAs in the bacterial cells is more efficient than by electroporation. It should be noted that, in the electroporation approach, co-transformation of DNAs occurs only under extreme parameters (e.g. a 2.5 kV pulse), not in routine DNA transformations (1.3–1.5 kV pulse). These extreme conditions for transformation might (by some unknown pathway) cause the low level of recombination occurring following electroporation.

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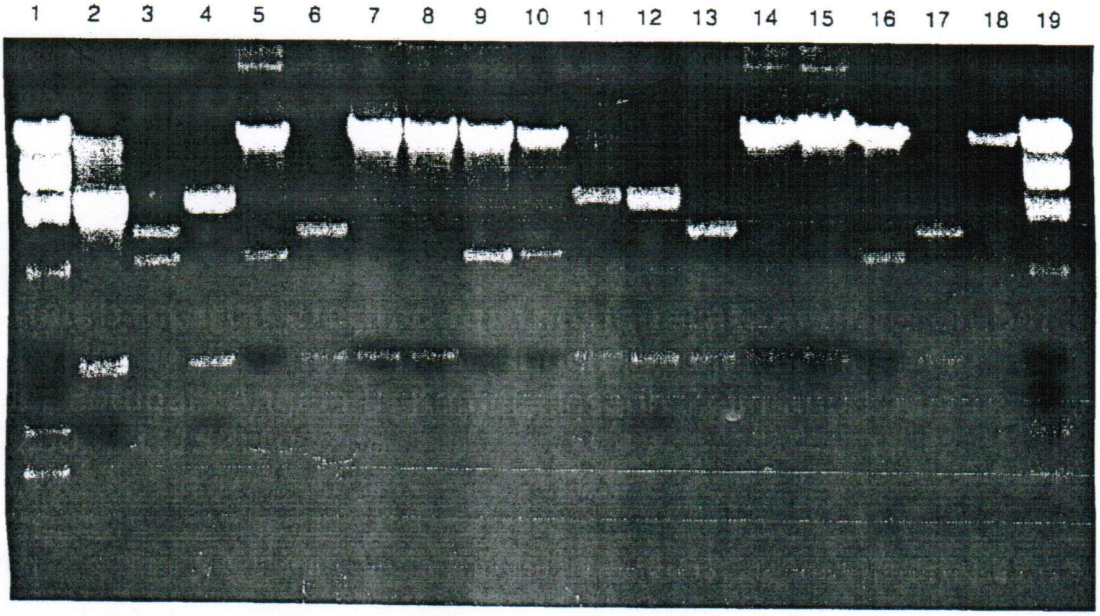
Fig. 1



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