Religation

Re-Ligation of Papillomavirus and Polyomavirus Genomes

Laboratory of Cellular Oncology, NCI

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Purpose: Most papillomavirus and polyomavirus molecular clones have been generated by ligating a restriction enzyme-digested episome into a bacterial maintenance plasmid. HindIII, EcoRI or BamHI have typically been used for incorporation into pBR322-based backbones. Molecular clones can be reconstituted by excision, followed by intra-molecular ligation. It is important that the re-ligation be performed under very dilute conditions, which favor intra-molecular ligation. Concentrated conditions favor unwanted inter-molecular ligation events since the diffusive random walk between the ends of neighboring DNA molecules becomes shorter than the random walk between the DNA molecule's own ends.

This protocol calls for re-ligating 30-50µg of cloned HPV DNA in a volume of 9ml. Although this may seem extremely dilute, it's actually a bit of a compromise. Under the described conditions ~10-20% of the HPV DNA forms unwanted concatomers (inter-molecular ligations).

Head-to-head contatomerization can be very problematic, since it can result promoters reciprocally aimed at one another. This can lead to transcription of cytotoxic double-stranded RNAs, particularly if the cloning site is in the HPV early region. In this case it may be beneficial to ligate <25µg of DNA in a 9ml volume, which results in <10% concatomer formation.

Since polyomaviruses are smaller than papillomaviruses, the random walk from end to end is shorter. It is therefore possible to achieve a high degree of intramolecular religation of 50µg of polyomavirus genomic DNA using this protocol.

Our group does not favor the introduction of linearized viral genomes into cells. We feel concerned that the cellular DNA repair machinery might leave small insertions or deletions during the process of splicing together the free DNA ends, thus mutagenizing the viral genome.

Step 1: cut out the plasmid backbone

•Use an appropriate restriction enzyme (BamH1 for pHPV16-114k, Nco1 for HPV18) to cut the backbone out of 50 µg of a papillomavirus plasmid in a 200 µl volume.

•Heat inactivate the restriction enzyme (if possible – see enzyme entry in the NEB catalog). If the restriction enzyme can't be heat inactivated (e.g., BamH1 in pHPV16-114k) purify the digested DNA. To purify, add 1200µl of Qiagen PB buffer to the digest and purify over two Qiaprep blue miniprep spin columns (25µg binding capacity each column). Follow the Qiagen PCR purification kit protocol (run sample through the column, wash with PE buffer, spin dry, then elute each column with 2x 100µl volumes of TE.

Step 2: ligate under dilute conditions

•Bring the digested DNA (30-50µg total) up to a 9 ml volume of 1x ligase buffer in a 50 ml conical tube. Add 6 µl of high concentration (2m U/ml) T4 DNA ligase (NEB). Ligate at 16º C overnight. Note: one hour of ligation is not adequate.

Step 3: Ethanol precipite the ligated DNA

(see Crouse & Amorese (1987) Invitrogen/BRL *Focus 9(2):3*)

•Add 4.5 ml of 7.5 M ammonium acetate (Sigma cat# A2706) and mix. Add 35 ml of 95%ethanol. Do not use absolute (100%) ethanol. Mix and incubate at 4ºC overnight.

•Bring the sample back to room temperature. Centrifuge at ~5,000 x g (room temp) for 60 minutes (e.g., Sorvall SH3000 swinging bucket rotor 4700 rpm).

•Wash pellet with 10 ml of 70% ethanol. Spin briefly. Repeat.

•Remove the second 70% ethanol wash. Spin residual ethanol off the walls of the tube. Remove residual ethanol, then allow pellet to air dry for several minutes.

•Add 100 µl of TE to the dried pellet. Allow the DNA to redissolve for at least 10 minutes. Transfer dissolved DNA to a microfuge tube. Rinse the walls and bottom of the 50ml tube using an additional 100 µl of water. Expect a yield of about 35µg of DNA.

Step 4: Analyze religated DNA by agarose gel electrophoresis

Expect to see supercoiled and relaxed circular bacterial backbone (~1.8 and ~3.2 kb) and supercoiled and relaxed circular (nicked) HPV genome (~6.2 and >16 kb). At least 50% of molecules will be relaxed circular. It may be helpful to also digest a microliter or two of the purified DNA with a restriction enzyme. These conditions are at the upper end of acceptable DNA concentration. Consequently, some inter-molecular ligation events (concatomers) will occur. Head to head concatomers will show up as predictable extra bands after restriction digestion, whereas head to tail concatomers have restriction digest patterns indistinguishable from properly religated HPV genome.