gels. The RF-DNA in total DNA is amenable to direct manipulation by restriction enzymes; we are able to clone MSV from total DNA extracts quite routinely. It is also easy to enrich total DNA extracts from infected plants for viral RF-DNA by one of several physical or chemical methods developed for the isolation of plasmid DNA, and so treatment of total DNA from infected tissues enriches for the plasmid-like covalently closed circular (ccc) RF-DNA. These techniques include the following:

1. Equilibrium centrifugation in ethidium bromide-CsCl density gradients to separate RF-DNA from genomic DNA ***(17)***. Ethidium bromide intercalates into DNA and thus confers on cccDNA a different buoyant density to linear or open circular DNA. This method requires large amounts of infected tissue, and so may be of somewhat limited practical use.
2. The Hirt method ***(18)*** originally developed for the isolation of polyomavirus DNA from animal cells. This method enriches for low-mol-wt DNA by precipitating high-mol-wt DNA in the presence of 1*M* NaCl and 1% SDS; we and others have used it for the isolation of geminivirus replicons from small amounts of callus tissue transfected with recombinant geminivirus constructs (Palmer, Willment, and Rybicki, unpublished results; **ref. *19***). A similar method has found use in the isolation of BGMV RF-DNA ***(20)***.
3. Enrichment for cccDNA by denaturation of chromosomal DNA in the presence of alkali ***(21–23)***.

For isolation of geminivirus RF-DNA, we routinely use the alkaline-lysis plasmid preparation of Ish-Horowitz and Burke ***(24)***: This makes use of the observation that there is a narrow range between pH 12.0 and pH 12.5 in which linear, but not cccDNA is denatured. We isolate total nucleic acids from infected leaf material by grinding the tissue in liquid nitrogen to break open cells, resuspending the powdered tissue in a DNA-extraction buffer, and extracting with phenol:chloroform, followed by precipitation with isopropanol or ethanol. The nucleic acid pellet is then treated exactly as if it were a plasmid preparation: The high-mol-wt chromosomal DNA is precipitated by alkali treatment and neutralization and is separated from the ccc RF-DNA by centrifugation. We also incorporate a further round of purification of RF-DNA by anion-exchange chromatography on commercially available resin columns from a plasmid isolation kit. The protocol for RF-DNA isolation outlined in this chapter therefore yields very clean, highly purified RF-DNA, which is suitable for mapping directly with restriction endonucleases (**Fig. 2**) and even for direct sequencing using specific primers (Rybicki and Wallace, unpublished). We have used it routinely for the isolation of RF-DNA of MSV and the phloem-limited Begomovirus abutilon mosaic virus (Jacobson and Rybicki, unpublished results).