Jul 01, 2025

Isolation of Circular Single-Stranded DNA from Total DNA in Grapevine Samples

DOI
https://dx.doi.org/10.17504/protocols.io.5jyl82o38l2w/v1

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Nanovirseq

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Protocol Citation: Vahid Jalali Javaran 2025. Isolation of Circular Single-Stranded DNA from Total DNA in Grapevine Samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5jyl82o38l2w/v1

Protocol status: Working
We use this protocol and it's working

Created: September 16, 2024

Last Modified: July 01, 2025

Protocol Integer ID: 107704

Keywords: epigenetic modifications of circular ssdna virus, length sequencing of the circular ssdna virus, native form of circular ssdna virus, circular ssdna virus, viruses from total dna extraction, purified viral dna, grapevine samples this protocol, viral dna, grapevine sample, total dna extraction, stranded dna, dna from total dna, dna, total dna, ssdna, detection of methylation pattern, genome structure, genome, epigenetic modification, methylation pattern

Abstract

This protocol details a method for the purification and enrichment of circular single-stranded DNA (ssDNA) viruses from total DNA extractions. Through a series of enzymatic reactions, the protocol is optimized to selectively enrich the native form of circular ssDNA viruses, preserving chemical modifications such as methylation on their genomes. The expected results include highly purified viral DNA, enabling full-length sequencing of the circular ssDNA virus and the detection of methylation patterns. This makes the protocol ideal for studying both the genome structure and epigenetic modifications of circular ssDNA viruses.

Guidelines

- Prepare all buffers and enzyme mixes fresh before use to ensure optimal enzyme activity.
- Use sterile, DNase-free water and tubes to avoid contamination of the DNA sample.
- Ensure accurate pipetting of enzymes and reaction components, as enzyme activity depends on precise reaction conditions.
- Incubation times and temperatures must be strictly followed for efficient digestion and processing of the DNA.
- Bead washing and elution steps should be performed gently to prevent DNA loss or damage.

Materials

Buffers and Solutions:

rCutSmart™ Buffer (10X) (Modified as NEBuffer™ 4 by adding DTT):

- 50 mM Potassium Acetate
- 20 mM Tris-acetate
- 10 mM Magnesium Acetate
- 100 µg/ml Recombinant Albumin
- **1 mM DTT** (added to create NEBuffer™ 4)
- pH 7.9 at 25°C

- **E. coli DNA Ligase I Buffer (10X):** Used for ligation reactions with DNA ligase and ploymerase activity with Klenow.
- Sodium Acetate (3 M, pH 5.2)
- Cold 75% Ethanol (pre-chilled at -20°C)
- 100% Ethanol
- DNase-free Water

Enzymes:Restriction Enzymes:

- SrfI (20,000 units/ml)
- SpeI-HF (20,000 units/ml)
- Nicking Endonuclease:Nt.AlwI (10000 units/ml)
- Exonucleases:T7 Exonuclease (Double-stranded DNA specific, 10,000 units/ml)
- Exonuclease T (Single-stranded DNA specific, 5,000 units/ml)
- E. coli DNA Ligase (10,000 units/ml)
- DNA Polymerase I, Large (Klenow) Fragment (5,000 units/ml)

Consumables:

- Microcentrifuge tubes (1.5 mL and 2 mL)
- Pipette tips (sterile, DNase-free)
- Magnetic beads for bead washing and DNA elution (AMPure XP Bead-Based Reagent)

Equipment:

- Centrifuge capable of speeds up to **15,700 g**
- Magnetic stand for bead washing
- Qubit fluorometer kit for DNA quantification
- PCR machine or thermal cycler
- Heating block or water bath (set to 37°C and 25°C as needed)

Safety warnings

- Always wear gloves and use a fume hood when handling enzymes, DTT, and other reagents to avoid contamination and exposure.
- Restriction enzymes and exonucleases are temperature-sensitive; ensure proper storage and handling to maintain activity.
- Dispose of all biohazardous waste, such as used tips and tubes, according to your institution's biosafety protocols.
- Bead washing steps must be performed carefully to prevent DNA loss; do not overdry or overwash the beads.
- Validate digestion efficiency (e.g., Qubit) before proceeding to ensure the protocol's success.

Before start

- Confirm the total DNA concentration is sufficient for the reaction (we used ~22 µg for initial digestion).
- Prepare the following buffers and enzyme mixes:
- **rCutSmart™ Buffer** for restriction enzyme digestion.
- ***E. coli* DNA Ligase Reaction Buffer** for polymerase activity.
- Preheat the incubators or water baths to **37°C** for restriction digestion and **25°C** for exonuclease reactions.
- Have bead washing and elution materials ready, ensuring the beads are appropriately equilibrated for efficient DNA recovery.

Double-Stranded Circular DNA Digestion

1 **Prepare the Reaction Mixture:**

- Combine the following in a microcentrifuge tube:

	Component	Volume	Details
	Total DNA	~	~22 µg total DNA extracted from grapevine samples
	rCutSmart Buffer (10X)	18 µL	Provides optimal conditions for restriction enzyme activity
	SrfI	3 µL	60 units, cuts specific dsDNA sites
	SpeI-HF	3 µL	60 units, cuts specific dsDNA sites
	Nt.AlwI	3 µL	30 units, introduces single-strand breaks into circular dsDNAs
	DNase-free water	Adjust to 180 µL	Final reaction volume

Explanation: This step digests double-stranded circular genomes and plasmids to linearize them, ensuring they can be degraded in the subsequent steps.
Point: The selection of restriction enzymes like **SrfI** and **SpeI-HF** in the protocol depends on the specific recognition sites present in the chloroplast and mitochondrial genomes of the plant species being studied.
Incubation: Incubate at **37°C for 2 hours** to ensure complete digestion.

Digestion of Linear Double- and Single-Stranded DNAs

2 This step removes all linear DNA fragments (both double-stranded and single-stranded) that were generated during the restriction digestion step. The remaining DNA will predominantly consist of circular single-stranded DNA (ssDNA).

	Component	Volume	Details
	T7 Exonuclease	3 µL	30 units; degrades **linear double-stranded DNA** efficiently
	Exonuclease T	5.1 µL	25 units; degrades **linear single-stranded DNA**
	DTT (10 mM)	1.9 µL	Final concentration 1 mM; stabilizes enzyme activity and prevents oxidation

- Add the components listed above to the reaction tube containing the digested circular dsDNA from the previous step.
- Mix thoroughly and ensure the reaction components are evenly distributed.
- Incubate at **25°C overnight** to allow complete degradation of both linear dsDNA and ssDNA fragments.

Explanation: This step leaves only circular DNA intact, ensuring that no linear DNA contaminants interfere with downstream applications. Circular single-stranded DNA (ssDNA) remains protected from exonuclease activity due to its structure.

Bead Washing and Elution

3 Do Bead washing to remove any residual contaminants and purifies the remaining DNA. In all bead washing steps performed in this protocol, 1.5X ratio of beads relative to the volume of the DNA solution was used to ensure efficient purification and recovery of DNA.

	Procedure	Details
	Bead Washing and Elution	Use magnetic beads to wash the sample, eluting the DNA in **85 µL**.
	Qubit and PCR Validation	Use **1 µL** of the eluted DNA to measure concentration and confirm digestion of linear GRBV DNA.
	Outcome	in our experiment, from **22 µg total DNA**, approximately **20 µg linear DNA** was digested, leaving **~2 µg** for the next step.

Point: This step is **very important**. Any residual enzymes (e.g., exonucleases or restriction enzymes) can degrade circular DNA during subsequent steps. Perform this step carefully to ensure complete removal of enzymes and buffers.

Synthesis of Complementary Strand of circular single-stranded DNAs (ssDNA)

4 This step synthesizes the complementary strand for circular single-stranded DNA (ssDNA), effectively creating double-stranded DNA (dsDNA).

	Component	Volume	Details
	Circular ssDNA (Digested DNA)	78 µL	Purified DNA from the previous step (2 µg)
	E. coli DNA Ligase I Buffer (10X)	10 µL	Provides optimal conditions for ligase activity and strand synthesis
	dNTP (10 mM)	4 µL	Supplies nucleotides for DNA synthesis

	Component	Volume	Details
	Klenow DNA Polymerase I	4 µL	Synthesizes the complementary strand, creating double-stranded DNA
	E. coli DNA Ligase	4 µL	Facilitates ligation of nicked dsDNA
	DNase-free water	Adjust to 100 µL	Final reaction volume

Set Up Reaction:

- Combine the above components in a clean microcentrifuge tube.
- Ensure the DNA, buffer, and enzymes are mixed gently to avoid damage to the DNA.
- Incubate the reaction at 16°C for 2.5 hour.

Point: During this incubation, Klenow DNA Polymerase I synthesizes the complementary strand of the circular ssDNA. E. coli DNA Ligase seals the nicks to form complete double-stranded DNA.

Bead Washing and Elution:

Use magnetic beads to purify the synthesized dsDNA.

Elute in an appropriate volume of DNase-free water (typically 50 µL).

Final Digestion with the restriction enzyme (KpnI)

5 **This step was performed to produce linear double-stranded DNA from the GRBV genome. One strand of the resulting DNA represents the native strand of the circular ssDNA, while the other strand is the complementary sequence synthesized during the Klenow reaction. The digestion by KpnI ensures the DNA is linearized, which is necessary because the Nanopore library preparation kits require linear double-stranded DNA as input material for sequencing.**

	Component	Volume	Details
	Circular dsDNA	~	Purified circular DNA from previous step
	KpnI-HF	3 µL	60 units
	10X NEBuffer	5 µL	Provides optimal conditions for KpnI activity
	DNase-free water	Adjust to 50 µL	Final reaction mixture

- Combine the components in a clean microcentrifuge tube.
- Incubate at **37°C for 1 hour** to allow digestion by KpnI.

- **Final Bead Washing (Critical Step):**
- Use magnetic beads to wash the reaction mixture thoroughly, ensuring all residual enzymes and buffer components are removed.
- Elute the purified linear DNA for further analysis.

Point:
We used **KpnI** because it is the one of restriction enzymes that cuts the GRBV genome at a single site, enabling precise linearization and having the full length of GRBV. Users of this protocol should select a restriction enzyme that cuts their viral genome at one specific site to achieve accurate linearization for downstream library preparation and sequencing.

- **Measure DNA Concentration**

Use Qubit to measure DNA concentration; in our case yield was ~4 µg.