bio-protocol

Analysis of Telomeric G-overhangs by in-Gel Hybridization

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[Abstract] Telomeric DNA in majority of eukaryotes consists of an array of TG-rich tandem repeats. The TG-rich DNA strand is oriented with its 3' end towards chromosome termini and is usually longer than its complementary CA-rich strand, thus forming 3' single stranded overhang (G-overhang). G-overhangs arise from incomplete replication of chromosome termini by the lagging strand mechanism and post-replicative nucleolytic processing. The G-overhang is important for telomere protection as it serves as a binding platform for specific proteins and is required for t-loop formation. Hence, structure of telomeric G-overhang is an important indicator of telomere maintenance and functionality. Here we describe a method for analysis of G-overhangs in a model plant *Arabidopsis thaliana* by in-gel hybridization technique. This method allows relative quantification of the amount of single stranded telomeric DNA. Short telomeric probes are radioactively labeled and hybridized to DNA under non-denaturing conditions to specifically detect ssDNA. Total telomeric DNA can be measured using denaturing conditions in the same gel and this procedure usually follows the non-denaturing in-gel hybridization. Terminal nature of the ssDNA is verified by exonuclease treatment. This technique was originally developed in yeast and now is used as a major tool for G-overhang analysis in a variety of organisms ranging from human to plants.

Materials and Reagents

1. Plastic wrap (common food wrap or Fisherbrand™ Clear Plastic Wrap) (Thermo Fisher Scientific, catalog number: 22-305654)
2. Whatman® papers (Sigma-Aldrich, catalog number: 3030917)
3. GeneJET Plant Genomic DNA Purification Kit (Life Technologies, catalog number: K0792)
   Note: Currently, it is "Thermo Fisher Scientific, catalog number: K0792".
4. T4 DNA polymerase (NEB, catalog number: M0203S)
5. NEB buffer #2 (NEB, catalog number: B7002S)
6. dNTPs (Life Technologies, catalog number: R0181)
   Note: Currently, it is "Thermo Fisher Scientific, catalog number: R0181".
7. Isopropanol
8. Absolute ethanol (EtOH)

9. HindIII (Thermo Fisher Scientific, catalog number: ER0501)

10. 3 M NaOAc (pH 5.2)

11. DNA Gel Loading Dye (6x) (Life Technologies, catalog number: R0611)

    Note: Currently, it is "Thermo Fisher Scientific, catalog number: R0611".

12. PeqGold Universal agarose (VWR, catalog number: 732-2789)

13. Ethidium Bromide (EtBr) solution (1%) (Applichem, catalog number: A1152)

14. T4 polynucleotide kinase (PNK) (10 U/µl) (Thermo Fisher Scientific, catalog number: EK0031)

15. Custom oligonucleotide probe, (TA3C3)4 or (TA3C3)3 (10 pmol/µl) (Sigma-Aldrich)

16. γ-^{32}P-ATP (> 6,000 Ci/mmol) (HARTMANN ANALYTIC GmbH, catalog number: SRP-501)

17. QIAquick Nucleotide Removal Kit (QIAGEN, catalog number: 28304)

18. Tris

19. Acetic acid

20. EDTA (pH 8.0)

21. NaCl

22. Sodium citrate (pH 7.0)

23. SDS

24. Na-phosphate buffer (pH 7.2)

25. BSA

26. Tris-Cl (pH 8)

27. 1x TAE (see Recipes)

28. 20x SSC (see Recipes)

29. Hybridisation buffer (see Recipes)

30. Wash solution-1 (see Recipes)

31. Wash solution-2 (see Recipes)

32. Denaturation solution (see Recipes)

33. Neutralization solution (see Recipes)

Equipment

1. Eppendorf™ ThermoMixer™ C

2. Centrifuge (Eppendorf AG)

3. Horizontal Midi Gel Electrophoresis system (PerfectBlue Gel System Midi S, Peqlab)

4. Gel Doc™ XR+ System (Bio-Rad Laboratories)

5. Gel dryer (Bio-Rad Laboratories, model: 583)

6. Imaging plate (Fujifilm, catalog number: BAS-IP MS 2025)

    Note: Currently, it is "GE Healthcare, catalog number: BAS-IP MS 2025".

7. Vacuum pump (Gardner Denver Thomas GmbH, ILMVAC, catalog number: 412711)

8. Water bath with shaking

9. Pharos FX Plus Molecular Imager (Bio-Rad Laboratories)
10. PC with an image analysis software (*e.g.,* Image Lab from Bio-Rad Laboratories)

Software

1. Image Lab 5.1 (Bio-Rad Laboratories) (freely available at http://www.bio-rad.com/en-us/product/image-lab-software)

Procedure

A. Non-denaturing in-gel hybridization
1. Genomic DNA was prepared using standard procedures. DNA was prepared as method described in Kazda *et al.* (2012) or using GeneJET Plant Genomic DNA Purification Kit. When 2x CTAB DNA extraction procedure (Borevitz *et al.*, 2003) was used, incubation was done at 55 °C (for 1 h) instead of 65 °C to reduce a risk of DNA melting.
2. Negative control was generated by removing terminal G-overhangs with T4 DNA polymerase that has a strong 3' exonuclease activity on ssDNA. Genomic DNA (2 µg) was treated with 30U of T4 DNA polymerase in presence of 0.1 mM dNTPs in total reaction volume of 200 µl of 1x NEB buffer #2. Reaction was incubated for 30 min at 37 °C. DNA was precipitated with 200 µl isopropanol, centrifuged 30 min at max speed at RT and washed with 70% EtOH. Resuspension of dry DNA was carried out at 37 °C in 175 µl dH$_2$O. Samples were processed immediately.
3. Samples and their T4 DNA polymerase pretreated negative controls (2 µg each) were digested with 50 U of HindIII in total reaction volume of 200 µl of 1x Buffer R. Reaction was incubated overnight at 37 °C. DNA was precipitated with 20 µl of 3 M NaAc (pH 5.2) and 200 µl of isopropanol, centrifuged 30 min at max speed at RT and washed with 70% EtOH. Resuspension of dry DNA was carried out at 37 °C in 20-25 µl dH$_2$O. Samples were stored at -20 °C or processed immediately and 4-5 µl of DNA Gel Loading Dye (6x) was added.
4. Digested DNA was separated using agarose gel electrophoresis. Gel was prepared in 1x TAE at a final concentration of 0.9% agarose. Samples were loaded and separated at 25 V for 15 h. DNA was stained by ethidium bromide. Gel was soaked in 200 ml of 2x SSC with 20 µl of 1% ethidium bromide at RT for 30 min while shaking.
5. DNA was visualized by UV and picture was acquired using Gel Doc™ XR+ System (Figure 1). Attention was paid that lanes were not over saturated (Figure 2, for accurate signal quantification).

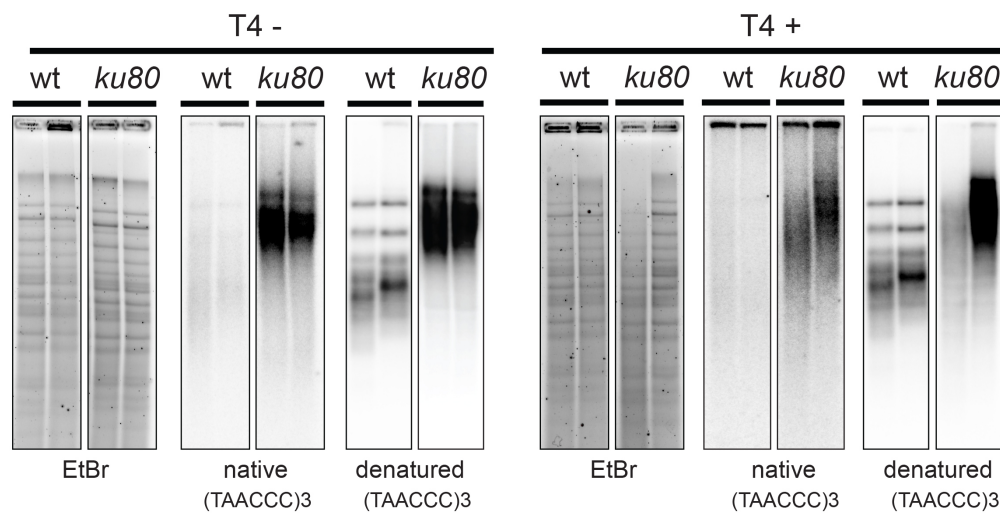**Figure 1. In-gel hybridization analysis of wild type and *ku80* mutant *A. thaliana*.** The digested DNA of two biological replicates of wild type (wt) and mutant (*ku80*) was separated on an agarose gel and hybridized with a radioactively labeled (TAAACCC) 3 probe first under native and then under denaturing conditions (left panel). The same procedure was used for T4 treated samples (right panel).
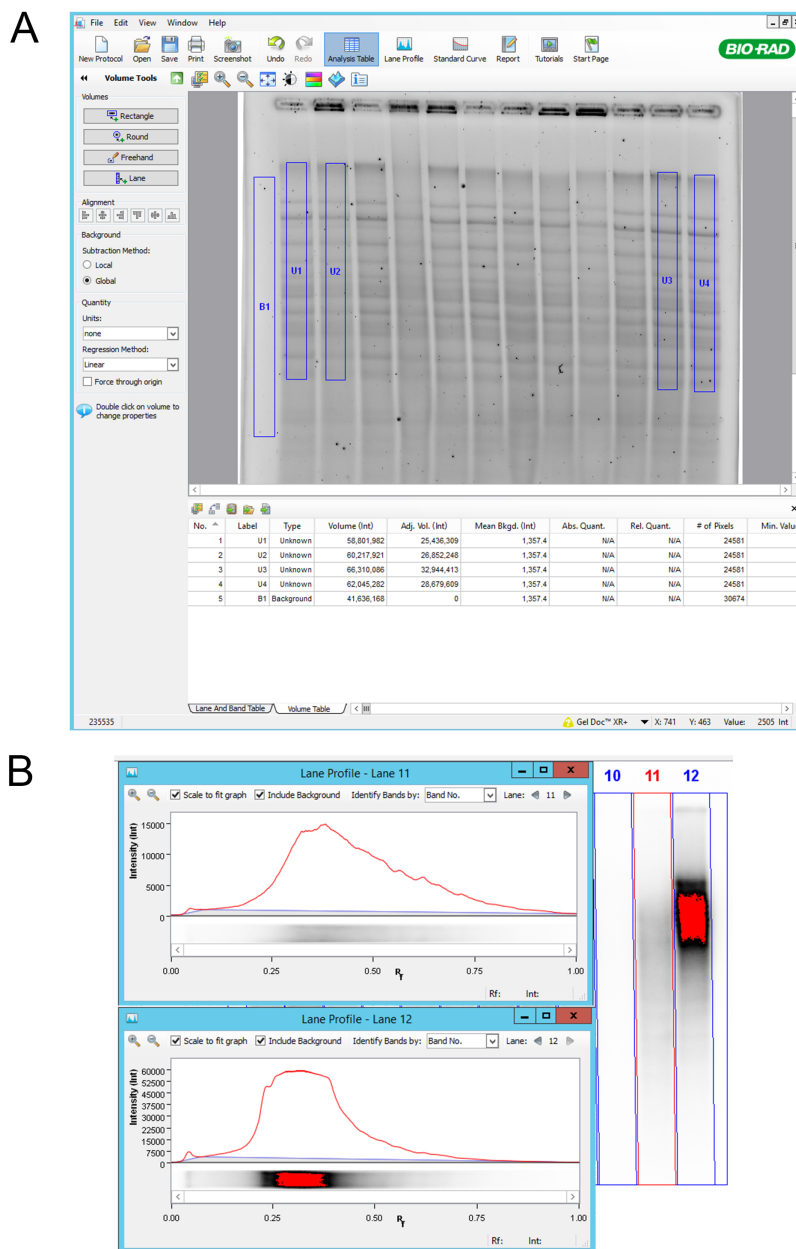
Figure 2. Volume analysis using Image Lab 5.1 software. A. "Rectangle"; Using "Rectangle" function in "Volume Tools", we selected relevant lane area and background. Background subtraction was set to Global and "Adj. Vol." values were exported for further analysis; B. Lane profile; Attention was paid that lanes were not showing the signal saturation. Saturation of the signal can be seen as red color in lane 12. In the lane profile we can see flattening of the signal peak where we cannot fully observe the total amount of the signal.

6. Agarose gel was dried using a gel drier. Gel was sandwiched between two Whatman papers and dried in a gel drier under vacuum at RT for 10 min per side. Gel was processed immediately.

7. For G-overhang detection, 10 pmol of (TA3C3) 4 or (TA3C3) 3 oligonucleotide probe resuspended in dH_2O was labeled in presence of 5 µl of γ-32P-ATP (> 6,000 Ci/mmol) using 10 U T4 PNK in total reaction volume of 20 µl of 1x PNK A Buffer. Reaction was incubated at 37 °C for 30 min and purified with QIAquick nucleotide removal kit according to manufacturer´s instructions.

8. Dried gel was prehybridized in 50 ml hybridization buffer (in a plastic container) in 50 °C water bath with shaking (35 rpm) for 1-2 h. A heavy item was used to fix the container on the bottom of the water bath, so it does not float. Entire volume of purified probe was added and hybridized overnight at 50 °C in the water bath with shaking (35 rpm).

9. Gel was removed from the hybridization solution and rinsed with wash solution-1 briefly, followed by two additional washes with wash solution-1 at RT, 35 rpm shaking for one hour. Later two washes with wash solution-2 at 38 °C, 35 rpm shaking for one hour was given.

10. Gel was dried at RT between Whatman papers in gel drier under vacuum. Attention was paid not to over dry the gel (maximum 1 min drying time in total).

11. Gel was wrapped in plastic wrap and exposed to imaging plate without a delay for up to 3 days at 4 °C to avoid contamination. Freezing and thawing causes gel to become brittle and was avoided.

12. Signal was detected on Pharos FX (Figure 1).

B. Denaturing in-gel hybridization

This procedure usually follows the non-denaturing in-gel hybridization to detect total telomeric DNA in analyzed samples.

1. Gel was unwrapped and DNA was denatured by soaking and rocking gel lightly in denaturing solution (100-200 ml) for 30 min at RT (35 rpm).

2. Denaturing solution was removed and gel was rinsed with dH_2O prior to adding neutralization solution (100-200 ml) for 15 min at RT.

3. Equilibration was done in 100-200 ml of 5x SSC for 15 min at RT and gel was prehybridized in 50 ml hybridization solution for 1 h as indicated previously.

4. Hybridization was done overnight with the same probe and conditions as was used for the non-denaturing in-gel hybridization.

5. Washing and exposure were done as for the non-denaturing in-gel hybridization. Shorter exposure (1 d) is usually sufficient for detecting strong signal (Figure 1).

C. Signal calculation by "Image Lab 5.1" software

The G-overhang signal from samples is normalized to DNA loading determined by ethidium bromide staining, and calculated by subtracting corresponding negative control pretreated with T4 DNA polymerase.
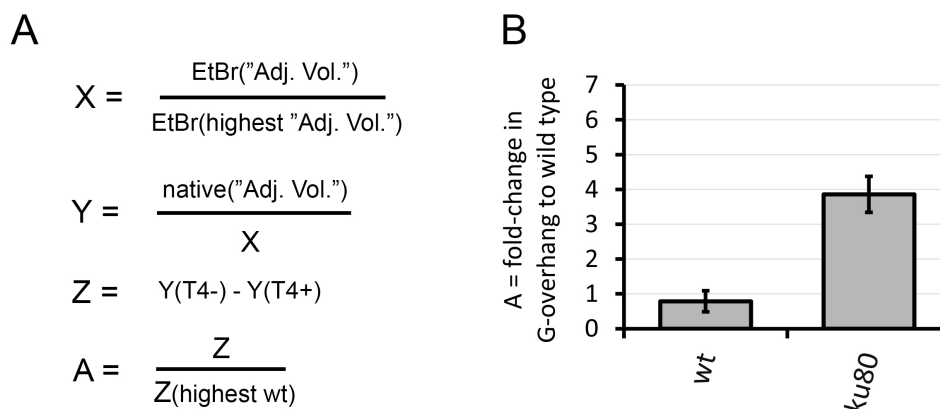
1. EtBr gel (loading control)

   a. "Volume tools" "Rectangle" was used for quantification.

      i. Selected area. Always the same rectangle was used for all lanes (first rectangle was copied and pasted). The area was selected in a way that the whole lane is covered as best as possible. However, the area around the lanes was not included in the rectangle (Figure 2A).

      ii. Selected background. The "Global" subtraction method was used and the rectangle was also used for this purpose (Figure 2A).

   b. "Analysis table" was used.

      i. Values were copied to clip board and transferred to excel. Attention was paid to decimal point. Adjusted values "Adj. Vol." were used (values corrected for background by the software Figure 2A).

      ii. Ratio was calculated (highest value "wt_high" was set to 1, other value "mut" - was corrected, Figure 3A).

      $X = $ EtBr ("Adj. Vol.") / EtBr (highest "Adj. Vol.")

      e.g. wt_high=5,000 and mut=3,000   -> X_{mut}= 3,000/5,000= 0.6

           -> X_{wt}= 5,000/5,000= 1.0

2. Native gel (G-overhang signal)

   a. Volume analysis was done as previously.

   b. Adjusted values were corrected for loading (T4 untreated and T4 treated samples, Figure 3A).

      $Y = $ native ("Adj. Vol.") / X

      e.g. mut= 4,500 and X_{mut}= 0.6   -> Y_{mut}= 4,500/0.6= 7,500

           wt = 5,000 and X_{wt}=1.0   -> Y_{wt}= 5,000/ 1.0= 5,000

   c. Values were corrected for T4 treatment. Values were subtracted from T4 treated sample.

      $Z = Y(T4-) - Y(T4+)$

      e.g. Y_{mut}= 7,500 and Y_{mut_T4}= 1,200   -> Z_{mut}= 6,300

           Y_{wt}= 5,000 and Y_{wt_T4}= 1,000   -> Z_{wt}= 4,000

3. Ratio calculation (set highest wild type control sample "highest wt" to 1, Figure 3A).

   $A = Z / Z(\text{highest wt})$

   e.g. Z_{wt}= 4,000 and Z_{mut}= 6,300   -> A_{mut}= 6,300/4,000= 1,575

        -> A_{wt}= 1.0

4. Graphical representation

   Relative values from the point 3 are used to present the result of the analysis in a graph (Figure 3B).

Figure 3. Graphical representation of the In-gel hybridization analysis. A. Signal calculation; Signal from the EtBr gel was used to create a loading ratio (X). Signal from the native gel hybridization was first corrected for DNA loading (Y) and signal from T4 treated samples was subtracted from not treated samples (Z). A fold change in G-overhang was calculated to the wt sample with the highest value of Z (A); B. Relative quantification of the G-overhang signal; The relative G-overhang signal (calculated ratio) is represented as average fold changes in G-overhang signal relative to wild type with highest signal.

Recipes

1. 1x TAE
   40 mM Tris
   20 mM acetic acid
   1 mM EDTA (pH 8)
2. 20x SSC
   3.0 M NaCl
   0.3 M sodium citrate (pH 7.0)
   Dilute accordingly
3. Hybridization buffer
   7% SDS
   0.25 M Na-phosphate buffer (pH 7.2)
   0.1 g/L BSA
4. Wash solution-1
   0.25 x SSC
   0.01% SDS
5. Wash solution-2
   0.25 x SSC
6. Denaturation solution

150 mM NaCl

0.5 M NaOH

7. Neutralization solution

150 mM NaCl

0.5 M Tris-Cl (pH 8)

Acknowledgments

References

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