bio-protocol

A SYBR Gold-based Label-free *in vitro* Dicing Assay

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Abstract

In Arabidopsis, DICER-LIKE PROTEIN 3 (DCL3) cuts the substrate pre-siRNA into a product siRNA duplex, encompassing one 23-nt strand and one 24-nt strand. To monitor the separation of the siRNA duplex with only 1-nt difference, we developed this protocol to evaluate the *in vitro* dicing activity of DCL3. The method can be applied for measuring the lengths of single-stranded RNA separated through denaturing urea polyacrylamide gel electrophoresis (urea PAGE), which are visualized by a label-free fluorescence SYBR Gold, and quantified in a multi-function imager. This label-free method is easy to conduct, has low cost, and lacks the hazard of the traditional radio-labeled method. This method can also be adapted to the other Dicers and small RNAs.

Keywords: Dicer, Dicing activity, Small RNA, Urea PAGE, SYBR Gold

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Background

Dicer family endonucleases play critical roles in small RNA (sRNA) biogenesis by cutting the substrate double-stranded RNA (dsRNA) into product dsRNA with defined lengths, of approximately 19–30 bp (Chen *et al.*, 2010; Borges and Martienssen, 2015). The substrate recognition of Dicer depends on the termini features of the dsRNA. Typically, Dicers prefer substrate dsRNA, with the guide strand possessing 5'-phosphate, and the complementary strand possessing a 1- or 2-nt 3'-overhang. The cutting of the two RNA strands is simultaneously conducted by the paired RNaseIII domains, towards the 3' of the guide strand and the 5' of the complementary strand, yielding asymmetric dicing. Depending on the different 3'-overhang lengths, the two strands of the product dsRNA could be with the same length or with minor differences in length (usually, only ~1–2-nt). In addition, the cleavage efficiencies of different Dicers vary considerably (Fukudome *et al.*, 2011; Hung and Slotkin, 2021). Therefore, a bench-based simple and easy-to-use method to monitor both the lengths and quantity of the single-stranded RNA (ssRNA) strands of the siRNA duplex is required. The method should be efficient in separating different 19–30-nt ssRNAs with single base resolution, and could be adapted to a quantifying system. In past decades, the dicing assay was usually conducted by using phosphorus-32 (^{32}P) to label the RNA termini, denaturing urea polyacrylamide gel electrophoresis (urea PAGE) to separate ssRNA strands, and autoradiography to visualize the ssRNA on PAGE, which is expensive, hazardous, and time-consuming (Adachi and Yu, 2014). To prevent the hazards linked to ^{32}P and to make the assay more convenient in a small-scale lab, we developed this label-free, fluorescent dye SYBR Gold-based *in vitro* dicing assay, based on several previously published protocols (Tuma *et al.*, 1999; Seo *et al.*, 2019). In this protocol, we firstly listed the details for the dicing reaction (Nagano *et al.*, 2014), using a plant dicer called DICER-LIKE PROTEIN 3 (DCL3), and for running urea PAGE (Adachi and Yu, 2014), which employs 6–8 M urea to denature secondary RNA structures, enables the distribution of the ssRNA on the gel according to molecular weight (Dodd and Hudson, 2007; Ellington and Chory, 2001), and is convenient for monitoring the length differences in ssRNAs (Summer *et al.*, 2009; Petrov *et al.*, 2013; Green and Sambrook, 2021). The bands of the ssRNAs are further stained by SYBR Gold, which is a label-free fluorescent dye (Tuma *et al.*, 1999; Seo *et al.*, 2019), scanned by an image recorder, quantified using the ImageJ software (Seo *et al.*, 2019; Gassmann *et al.*, 2009; Schneider *et al.*, 2012), and statistically analyzed by the Origin 7.0 software. Considering the conserved mechanism of Dicer family enzymes and length distributions of sRNA, the protocol presented here can be adapted to dicing assays for most Dicer proteins, as well as other analysis regarding the length and quantification of sRNAs.

Materials and Reagents

1. 1.5 mL microcentrifuge tubes (Axygen, catalog number: MCT-150-C)
2. MgCl$_2$ (BBI Life Sciences, catalog number : A601336-0500)
3. HEPES (BBI Life Sciences, catalog number: A600264-0250)
4. KOH (Sangon, catalog number: A610441)
5. NaCl (Sigma, catalog number: S9625)
6. DTT (VETEC, catalog number: V900830)
7. Purified DCL3 protein (self-prepared in a buffer of 150 mM NaCl, 5 mM MgCl$_2$, 2 mM DTT, and 50 mM HEPES-KOH, pH 8.0)
8. RNA oligos (Takara)
9. Urea (Sigma, catalog number: U1250)
10. 40% Acrylamide/bis-acrylamide solution (19:1) (Sangon, catalog number: B546012-0500)
11. N,N,N',N'-tetramethylethylenediamine (TEMED) (Amresco, catalog number: M146)
12. Ammonium persulfate (APS) (Amresco, catalog number: M133)
13. Boric acid (Energy Chemical, catalog number: A060316)
14. Tris base (VETER, catalog number: WXBD5491V)
15. EDTA (Sangon, catalog number: E671001)
16. DEPC water (Sangon, catalog number: B501005)
17. 2× RNA Gel-Loading Buffer (Sangon, catalog number: B300596)

18. SYBR^{TM} Gold (Invitrogen, catalog number: S11494)
19. RNA Marker (Takara, catalog number: 3416)
20. RNaseZap (Invitrogen, catalog number: AM9780)
21. Reaction buffer (50 mL) (see Recipes)
22. 10× TBE (1 L) (see Recipes)
23. Denaturing gel mix (500 mL) (see Recipes)
24. Running buffer (1 L) (see Recipes)

Equipment

1. 20 cm × 20 cm glass plates (Beijing Liuyi Instruments, custom designed)
2. 1-mm thick spacers (Beijing Liuyi Instruments, custom designed)
3. Gel-sealing tape
4. Gel comb (Beijing Liuyi Instruments, custom designed)
5. Binder clips
6. Hi-temperature Dry Bath (Yooning, catalog number: YN-GO2-156)
7. Centrifuge (Eppendorf, Centrifuge 5424R)
8. High voltage electrophoresis system (BIO-RAD, PowerPac^{TM} HV Power Supply)
9. Gel electrophoresis chamber (Beijingliuyi, catalog number: DYCZ-22A)
10. Multi-function Imager (GE Healthcare, Amersham Imager 600)
11. Orbital Shaker (Yooning, catalog number: CS-200)

Software

1. ImageJ (https://imagej.nih.gov/ij)
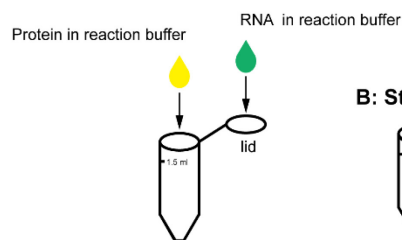2. Origin 7.0 (http://www.originlab.com)

Procedure

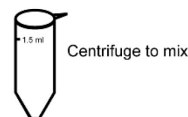### A. Dicing reaction (see Figure 1)

1. The recombinant soluble N-terminal strep-tagged AtDCL3 was expressed in the insect cell line sf9, using a Bac-to-Bac system (Invitrogen), and purified by Strep (IBA Life Science), Mono-Q (GE Healthcare), and Superdex G200 columns (GE Healthcare), as described (Wang *et al.*, 2021).
2. The purified protein was buffer changed to 150 mM NaCl, 5 mM MgCl$_2$, 2 mM DTT, and 50 mM HEPES-KOH, pH 8.0, concentrated to 1 mg/mL, aliquoted to 10–20 μL/tube, freezed by liquid nitrogen, and stored in -80°C. Before the dicing reaction, the tube containing freezed protein was placed on ice for 30 min, to allow the protein to melt.
3. Mix the 30-nt guide ssRNA (5'-phos-AACAAGCGAAUGAGUCAUUCAUCCUAAGUC) and the 31-nt complement ssRNA (GACUUAGGAUGAAUGACUCAUUCGCUUGUUC) in one tube, at a molar ratio of 1:1, in water. Anneal the two ssRNA into dsRNA by heating the mixture to 95°C, in a Hi-temperature Dry Bath for 1 min. Place the tube at room temperature to slowly cool-down. Depending on different Dicers and experimental designs, the RNA length and sequence vary accordingly.
4. Prepare 0.01 nmol dsRNA in 10 μL of reaction buffer (on the lid) and 0.003 nmol protein in 40 μL of reaction buffer (in the tube), separately (the ratio of protein and RNA may differ on different enzymes).
5. Spin all the tubes softly at the same time, to mix the protein and RNA to initiate reactions simultaneously.
6. Put the mixture into an incubator at 37°C for 5 min. The reaction time can be set from 1 minute to several hours, depending on different enzymes and experimental purposes.

7. Add 50 µL of 2× RNA loading buffer to the tube lid. Then, spin all the tubes at the same time to transfer the loading buffer into the reaction mixtures, to stop reactions simultaneously.
8. Heat the sample to 94°C for 5 min, and quickly transfer it onto the ice for 10 min.
9. Spin the ice-cooled sample tubes with an Eppendorf Centrifuge 5424R at 13,500 × *g* and 4°C for 1 min.
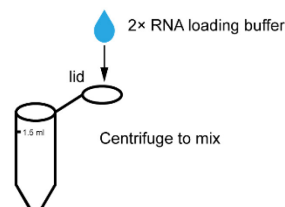10. Each experiment should be repeated three times.

A: Step 1 Prepare the protein and RNA separately

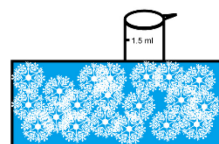Protein in reaction buffer   RNA in reaction buffer

lid

B: Step 2 Incubat the mixture at 37 °C

Centrifuge to mix

E: Step 5 Cooling the sample

C: Step 3 Prepare sample for page gel

2× RNA loading buffer

lid

Centrifuge to mix

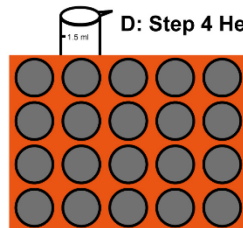**D: Step 4 Heat the sample in 94 °C for 3-5 min**

Figure 1. Workflow for the dicing reaction and sample preparation

B. Preparation of the urea PAGE gel (Adachi and Yu, 2014; Green and Sambrook, 2021; Rio *et al.*, 2010; Summer *et al.*, 2009).

1. Treat the gel plates with RNaseZap, then wash them with RNase-free water.
2. Lay the larger glass plate on the bench top and arrange the 1 mm spacers on both sides, but not the top and bottom of the glass plate. Cover the second glass plate to assemble the 20 × 20 cm gel plates with 1 mm spacers.
3. Seal three slides of the gel plates with gel-sealing tape, to form a sealed slit with a thickness of 1 mm (longer and thinner gels lead to better resolution).
4. Clamp the plates on both sides with eight or nine binders (three on each side, and two or three on the bottom), to clamp the gel plates and spacers together.
5. Prepare 34.65 mL of 15% acrylamide gel mixture (see Recipe) in a 50 mL tube.
6. Add 350 µL of APS into the 50 mL tube, and quickly and carefully mix the solution, to prevent air bubbles.
7. Add 10 µL of TEMED into the 50 mL tube, and quickly and carefully mix the solution, to prevent the air bubbles.
8. Pour the mixture into the slit (Note: avoid air bubbles), insert the 26-holes comb, and wait for 30 min, to allow the gel to polymerize.
9. Remove the comb and the tape, and mount the gel plates onto the gel running apparatus. Add 0.5× TBE to both the upper and lower reservoirs. Clamp an aluminum plate to the front side of the gel plate.

10. Pre-run the gel at 5 W for 60 min, to equilibrate and preheat the gel.

C. Running the gel
1. Rinse the wells extensively, until they are as clear as possible, with 0.5× TBE, using a syringe needle.
2. Load the samples into the wells.
3. Run the gel with a consistent power of 5 W for 3.5–4 h.

D. Visualizing the RNA stained by SYBR Gold
1. Remove the gel plates from the gel running apparatus. Use a metal spatula to pry the top glass plate off the gel.
2. Add water in a box. Carefully, flip the gel to face the water. Transfer the gel into the box, using water force, by shaking the box.
3. Remove the water from the box and add 30 mL of 0.5× TBE into the box, to submerge the gel.
4. Add 3 µL of 10,000× SYBR Gold into the box in a dark room, and shake the gel on a shaker at 50 rpm for 40 min.
5. Wash the gel with RNase-free water.
6. Place the gel on a fluorescent plate of the multi-function Imager, to visualize the RNA at the wavelength of 302 nm. The gel image can be stored in .tiff format.

E. Calculate the dicing activity by ImageJ (see Figure 2) (Wang *et al.*, 2021)
1. Download the ImageJ program from the website https://imagej.nih.gov/ij.
2. Go to "File" tab, click "Open" to load the gel image into ImageJ, and invert the background and the bands, which will show the image as black background and white bands.
3. Go to the "Analysis" tab, click "Set measurements", check the "Integrated Density" box, and then click the "OK".
4. Measure the raw integrated density (RID) of the background without any band, as that which exists inside a rectangle which can encircle the biggest band.
5. Measure all of the target bands separately. The resulting data file is then saved as an Excel spreadsheet.
6. Each relative RID value for each band is calculated by subtracting the measured RID value by the measured background RID.
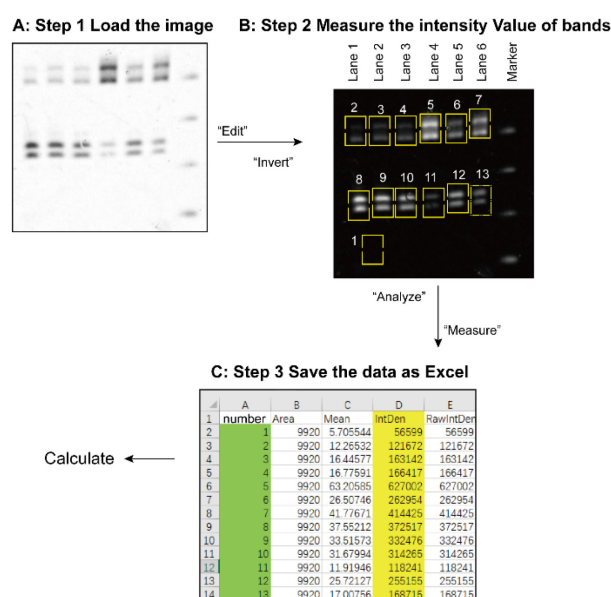

Figure 2. Workflow of ImageJ processing

Step 1, load the gel image into the ImageJ program. Step 2, invert the background and the bands. Lanes 1–6 represent the six samples. Band 1 shows the rectangle circle without any band inside, which is used as background area. This rectangle should be big enough for encircle the biggest band. Bands 2–7 are the substrate that has not been cleaved. Bands 8–13 are the dicing products for the corresponding samples. Step 3, the data was saved as an Excel file. The relative RID value of each sample band is calculated as the measured RID value of the band minus the measured background RID value from band "1".

The dicing activity of each sample is presented by the dicing activity of the relative RID of the lower band of the panel divided by the sum of the lower and the upper bands of the same panel, then multiplying this by 100. This workflow only represents a single time processing. Three replicates of the experiment should be conducted. The standard deviation (SD) value of the three replicates for one sample should be calculated.

F. Statistical analysis in Origin 7.0
1. Prepare the Origin 7.0 program (http://www.originlab.com).
2. Input the calculated values of interest for the three experimental replicates into the Origin program.
3. Go to "Statistics" tab, click "Hypothesis Testing"-"Two sample t-test" to analyze the difference between two samples.
4. Input the different P-value related to the specific sample, mark the value of $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.0001$ as " * ", " * * ", " * * * ", " * * * * ".
5. Go to "column" table, "set as" the "sample name", "mean value", "SD", " * ", corresponding to "X", "Y", "Y Error", "Label", separately.
6. Go to "Plot" tab, click "Column/Bar/Pie" to draw a corresponding statistic bar chart (Figure 3).
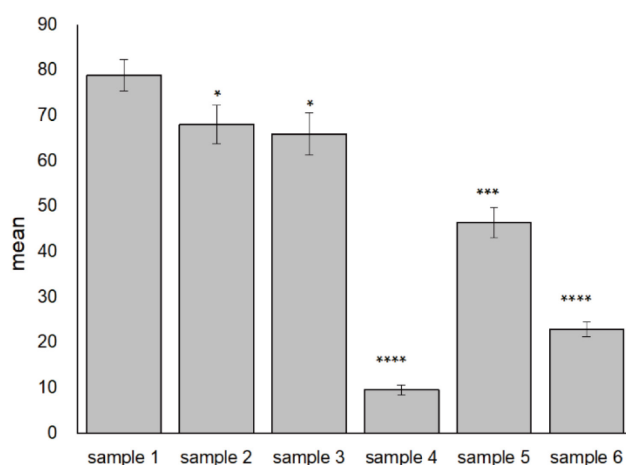

Figure 3. Example for *In vitro* Dicing Assay result by Origin.
The x-axis states the different samples, the y-axis shows the mean value of the dicing ratio with error bars presenting the SD. The dicing activity of samples 2–6 are lower than that of sample 1, with significant differences.

Notes

All of the water used in these procedures was RNase-free water. Every step should prevent contamination with RNase.
The SYBR Gold stain should be kept in the dark.

Recipes

1. Reaction buffer (50 mL)

Reagent	Final concentration	Amount
NaCl (5 M)	150 mM	1.5 mL
HEPES-KOH (1 M, pH 8.0)	50 mM	2.5 mL
$MgCl_2$ (1 M)	5 mM	0.25 mL
DTT (1 M)	2 mM	0.1 mL
Deionized water	n/a	45.65 mL

2. 10× TBE (1 L)

Reagent	Final concentration	Amount
Tris base	890 mM	108 g
EDTA	20 mM	6 g
Boric acid	890 mM	55 g
Deionized water	n/a	Add to 1 L in total

3. Denaturing gel mix (500 mL)

Reagent	Final concentration	Amount
10× TBE	1×	50 mL
40% Acrylamide/bis-acrylamide (19:1)	15%	187.5 mL
Urea	8 M	240.24 g
Deionized water	n/a	Add to 500 mL in total

4. Running buffer (1 L)

Reagent	Final concentration	Amount
10× TBE	0.5×	50 mL
Deionized water	-	950 mL

Acknowledgments

Competing interests

The authors declare no conflicts of interest or competing interests.

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