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Chapter 5

Primary MicroRNA Processing Assay Reconstituted Using Recombinant Drosha and DGCR8

Ian Barr and Feng Guo

Abstract

In animals, the Microprocessor complex cleaves primary transcripts of microRNAs (pri-miRNAs) to produce precursor microRNAs in the nucleus. The core components of Microprocessor include the Drosha ribonuclease and its RNA-binding partner protein DiGeorge critical region 8 (DGCR8). DGCR8 has been shown to tightly bind an Fe(III) heme cofactor, which activates its pri-miRNA processing activity. Here we describe how to reconstitute pri-miRNA processing using recombinant human Drosha and DGCR8 proteins. In particular, we present the procedures for expressing and purifying DGCR8 as an Fe(III) heme-bound dimer, the most active form of this protein, and for estimating its heme content.

Key words RNA processing, DiGeorge syndrome, Heme, RNA-binding protein, Nucleic acid-binding protein, Pasha, Ribonuclease III

1 Introduction

1.1 The Microprocessor Complex

The Microprocessor complex minimally contains the proteins Drosha [1], an RNase III family member, and DGCR8 [2, 3], an RNA-binding protein that also contains the cofactor heme [4–6]. Drosha and DGCR8 are essential for processing of all canonical microRNAs (miRNAs) in animals [7–9]. They are also sufficient to reconstitute pri-miRNA processing activity in vitro [3, 10]. Furthermore, quite a few proteins have been shown to regulate the Drosha/DGCR8-mediated cleavage of pri-miRNAs [11–18].

The domain structures of Drosha and DGCR8 have been dissected in several studies. Drosha contains two RNase III domains and a double-stranded RNA-binding domain (dsRBD) in the C-terminal region (Fig. 1a). The central region of Drosha, including residues 390–900, is highly conserved and required for pri-miRNA processing but contains no recognizable sequence motifs [10, 19]. DGCR8 contains a heme-binding domain, two dsRBDs, and a C-terminal tail (CTT) (Fig. 1b). The dsRBDs contribute to pri-miRNA binding [4, 20, 21]. The C-terminal tail has been

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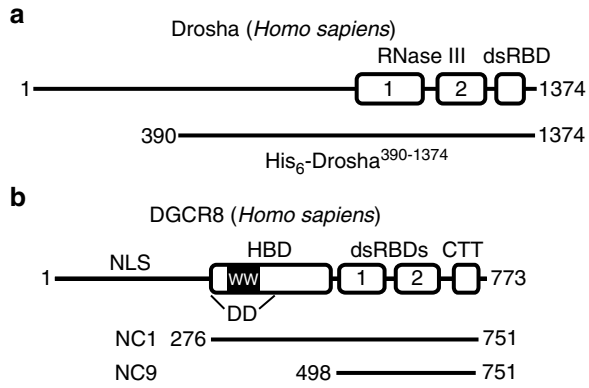

Fig. 1 Domain structures and recombinant expression constructs of human Drosha and DGCR8. The heme-binding domain (HBD) of DGCR8 includes a dimerization (sub)domain (DD)

shown to be important for co-immunoprecipitation of DGCR8 with Drosha in human cells [10] and for formation of proper higher order structure of DGCR8 upon binding pri-miRNAs [22]. See a recent review for more background information [23].

pri-miRNA processing may be analyzed in vitro using either whole-cell or nuclear extracts [24, 25], affinity-purified Microprocessor complexes expressed in mammalian cells [19, 26–28], or recombinant Drosha and DGCR8 expressed in heterologous systems [3, 10]. We focus on the last method in this review. Insect cell and bacterial expression systems allow active Drosha and DGCR8 proteins to be expressed with high yield and be purified to near homogeneity. These highly purified proteins do not contain other human proteins typically found to associate with Drosha and DGCR8 and enable the investigation of pri-miRNA processing with greater control of the experimental conditions.

Gregory, Shiekhattar, and colleagues were the first to show that Microprocessor may be reconstituted by expressing Drosha in insect cells and DGCR8 in *E. coli* [3]. The N-terminal 275 amino acids [10, 20] and the C-terminal 22/23 residues [4, 20] of the 773-residue DGCR8 have been shown to be dispensable for in vitro pri-miRNA processing. A DGCR8 construct with these residues deleted (named NC1, Fig. 1b) is the most active form in pri-miRNA processing in vitro, whereas a further truncation called NC9 (Fig. 1b), containing only the two dsRBDs and the CTT, is less active than NC1 [6]. The procedures for expression and purification of NC1 are described in this review.

1.2 DGCR8 as a Heme Protein

In addition to being an obligate partner of Drosha, DGCR8 also binds heme [4]. The central region of DGCR8, including the WW motif, encodes a unique dimeric heme-binding domain [29]. Each DGCR8 dimer binds one heme molecule [4]. The heme in native DGCR8, expressed in *E. coli*, is in the Fe(III) redox state [5].

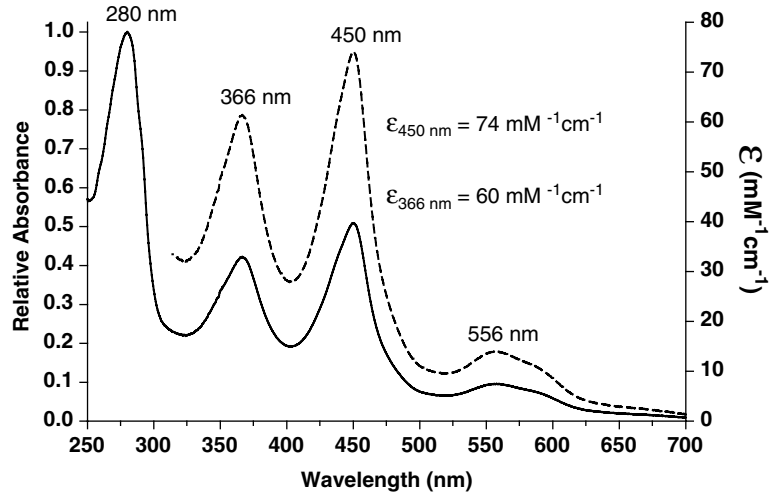

Fig. 2 Electronic absorbance spectrum of Fe(III) heme-bound NC1 dimer. The *solid line*, corresponding to the *left y*-axis, shows the relative absorbencies of the heme and protein peaks. The *dashed line*, corresponding to the *right y*-axis, shows the extinction coefficients of the heme, as determined using the pyridine hemochromagen assay as recently reported [30]

DGCR8 ligates to the Fe(III) using two Cys352 side chains contributed by both subunits; this coordination configuration results in characteristic absorption peaks at 366, 450, and 556 nm (Fig. 2) [5]. Recently, we show that Fe(III) heme activates dimeric apoNC1 for pri-miRNA processing in vitro, whereas Fe(II) heme does not [6]. Dimerization and heme binding are likely conserved properties of DGCR8 in all vertebrates and at least some invertebrates such as the star fish *Patiria miniata* [30]. Heme and the heme-binding domain appear to be important for pri-miRNA processing both in vitro and in vivo ([4, 6, 29] and our unpublished data), though their physiological functions have not been determined.

In order to obtain consistent results in studying pri-miRNA processing and to interpret them properly, it is important to express and purify recombinant DGCR8 protein with optimal heme content. When NC1 is overexpressed in *E. coli*, a heme-deficient condition is generated and hence some heme-free protein is produced [4]. The heme-free NC1 may appear as dimer and monomer [4, 6]. At least a part of the latter species is actually heterodimer of NC1, in which a subunit is cleaved by bacterial proteases during overexpression and/or purification so that only a small fragment (the dimerization domain) is left bound to the intact subunit [29]. The heme content of DGCR8 is indicated by the $A_{450\ nm}/A_{280\ nm}$ ratio, if the protein is purified to be free of nucleic acids from expression hosts. It is also possible to prepare apoNC1 from Fe(III) heme-bound NC1 via reduction and heme removal for studying activation of DGCR8 by heme [6]. However, the preparation of apoNC1 is beyond the scope of this chapter.

1.3 Preparation of Recombinant Drosha Protein

Full-length Drosha expressed in insect cells has been used in several studies [3–5, 22, 29]. However, in our experience the full-length His_6-Drosha is poorly soluble and cannot be purified using either Ni affinity or ion-exchange chromatography; and only a small amount of partially purified active His_6-Drosha may be obtained using size exclusion chromatography [4]. The N-terminal 390 amino acid residues of Drosha are dispensable for in vitro activity [10]. Recently, we found that truncation of this region greatly improves the solubility of Drosha without compromising the activity [6]. The procedure for purifying His_6-$Drosha^{390–1374}$ is described below.

1.4 Design of pri-miRNA Constructs for Reconstituted pri-miRNA Processing Assay

miRNAs may reside in introns or exons, messenger RNAs or independent transcripts [31]. Processing of pri-miRNAs by Microprocessor occurs co-transcriptionally [32]. Intronic pri-miRNAs may be processed by Drosha before splicing catalysis [33]. The exact 5′ and 3′ ends of pri-miRNAs at the time of processing are often not known. Biochemical studies show that pri-miRNA fragments containing the precursor miRNA (pre-miRNA) and certain lengths of the immediate flanking regions can be processed by Drosha and DGCR8 [1, 24]. The minimal lengths of the flanking regions for efficient processing by affinity-purified Microprocessor complexes may be as short as 10–20 nt [10, 19]. For reconstituted pri-miRNA processing assays, we typically include 30–60 nt on both sides of the pre-miRNA region.

The pri-miRNA fragments are typically prepared using T7 or SP6 RNA polymerase. The protocol for how to use T7 RNA polymerase is provided here. The transcription template should contain the T7 promoter, followed by the pri-miRNA coding sequence. For high transcription yields, the first two nucleotides of the transcript should be guanosines [34]. Either a PCR product or a linearized plasmid may serve as the template for the run-off transcription, in which the 3′-end of the RNA is roughly defined by the end of the template where the RNA polymerase simply falls off. The T7 RNA polymerase is known to add 0–3 non-templated residues at the 3′-end of the transcripts [34]. In the case where a plasmid template is used, a cleavage site for a restriction endonuclease such as *PstI* or *EarI* is engineered for linearization.

2 Materials

All solutions should be, to the greatest extent possible, free from RNase contamination. This applies especially to the reagents and buffers involved in transcription and pri-miRNA processing reactions and in the storage of RNAs.

2.1 Expression and Purification of DGCR8

1. The NC1 expression plasmid contains the coding sequence of amino acid residues 276–751 (NCBI accession no. of full-length DGCR8 cDNA: BC037564) inserted between *NdeI*

and *EcoRI* sites of pET-24a(+) (kanamycin resistant) or pET-17b (ampicillin resistant) vector. The PCR primers used in cloning are CAGCCATATGGATGGAGAGACAAGTGTGC (forward, the *NdeI* site underlined) and GCTCGAATTCAC TTTCGAGTCTCCTCCCT (reverse, the *EcoRI* site underlined).

2. *E. coli* strain BL21-CodonPlus (DE3)-RIPL (Agilent Technologies).
3. LB-Miller medium.
4. UV-visible absorption spectrophotometer equipped with a turbidity cuvette holder.
5. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
6. δ-aminolevulinic acid (δ-ALA).
7. High-speed centrifuge.
8. Sonics Vibra-Cell VCX 750 ultrasonic processor equipped with a standard probe.
9. Chromatography systems such as ÄKTA Purifier and ÄKTA Prime.
10. 5-mL HiTrap SP HP cation exchange column (GE Healthcare).
11. Superdex 200 10/300 GL gel filtration column (GE Healthcare).
12. DGCR8 lysis buffer: 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) (*see* **Note 1**).
13. DGCR8 buffer *A*: 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM DTT.
14. DGCR8 buffer *B*: 20 mM Tris–HCl pH 8.0, 2 M NaCl, 1 mM DTT.
15. SEC buffer: 20 mM Tris–HCl pH 8.0, 400 mM NaCl, 1 mM DTT.
16. Centrifugal concentrator with a molecular weight cutoff of 30 kDa.

2.2 Expression and Purification of Drosha

1. The His_6-$Drosha^{390–1374}$ expression plasmid has the coding sequence of amino acid residues 390–1,374 of human Drosha (NCBI accession no. of full-length cDNA: NM_013235) inserted between *BamHI* and *NotI* sites of pFastBac-HTb vector. The PCR primers used in cloning are CGCGGATCC AAAGAGCCCGAGGAGACC (forward, the *BamHI* site underlined) and GAGGATTAGAGCGGCCGCTTATTTCT TGATGTCTTCAGTCTC (reverse, the *NotI* site underlined). The recombinant His_6-$Drosha^{390–1374}$ contains a His_6-tag and a TEV cleavage site at its N-terminus (*see* **Note 2**).
2. Sf9 insect cells, culture medium, and transfection reagent.
3. Equipment same as the ones described for DGCR8 purification.

4. Ni Sepharose High Performance column (GE Healthcare).
5. Drosha lysis buffer: 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole, 20 % (v/v) glycerol, and 0.83 mM PMSF.
6. Drosha wash buffer: 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 10 mM imidazole, and 20 % (v/v) glycerol.
7. Drosha elution buffer: 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 200 mM imidazole, and 20 % (v/v) glycerol.
8. Drosha storage/reaction buffer: 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 10 % (v/v) glycerol.
9. Liquid nitrogen or dry ice–ethanol mix.

2.3 Transcription and Purification of pri-miRNAs Uniformly Labeled with ^{32}P

1. The transcription template for a human pri-miR-30a fragment is a linearized pUC19 plasmid containing the sequence GAATTC*TAATACGACTCACTATA***GGAAAG AAGGTATATTGCTGTTGACAGTGAGCGACTGT AAACATCCTCGACTGGAAGCTGTGAAGCCACA GATGGGCTTTCAGTCGGATGTTTGCAGCTGC CTACTGCCTCGGACTTCAAGGGGCTACTTTA GGAGCAATTATCTTGTTT**CgaagagTCTAGA (the *EcoRI* and *XbaI* cloning sites are underlined; the T7 promoter is in plain italic; the *EarI* site used for linearization is in lower case; and the coding sequence for the RNA is bold).
2. 10× T7 transcription buffer: 400 mM Tris–HCl pH 8.0, 250 mM $MgCl_2$, 40 mM DTT, 20 mM spermidine.
3. 10× NTP mix: 20 mM ATP, 20 mM GTP, 20 mM CTP, and 5 mM UTP.
4. [α-^{32}P] UTP (6,000 Ci/mmol, 10 mCi/mL).
5. T7 RNA polymerase.
6. Temperature-controlled dry bath and heat block.
7. 2× RNA loading dye: 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3), 10 M urea, 10 mM EDTA, 0.002 % bromophenol blue, and 0.002 % xylene cyanol.
8. Electrophoresis system, including glass plates (20×20 cm), spacers and combs (typically 0.75 or 0.80 mm thick), gel-running apparatus, and a power supply.
9. Denaturing 15 % polyacrylamide gel solution (50 mL): 1× TBE, 7 M urea, 18.75 mL 40 % acrylamide (acrylamide:bis-acrylamide 29:1) stock solution.
10. Tetramethylethylenediamine (TEMED), 500 μL.
11. 10 % (w/v) ammonium persulfate (APS).
12. Autoradiography film.

13. 1× TEN buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 100 mM NaCl.
14. Tube rotator.

2.4 Reconstituted pri-miRNA Processing Assays

1. RNaseOUT recombinant ribonuclease inhibitor.
2. Dry bath and electrophoresis system: Same as above.
3. Gelbond PAG film (Lonza).
4. Gel dryer, cold trap, and vacuum pump.
5. Storage phosphor screen.
6. Phosphorimager such as the Typhoon 9410 Variable Mode Imager (GE Healthcare).
7. Image analysis programs such as Quantity One (Bio-Rad), ImageQuant (GE Healthcare), or ImageJ (NIH, free online).

3 Methods

3.1 Expression of Heme-Bound DGCR8 NC1 in E. coli

1. Transform the NC1 expression plasmid to *E. coli*. Spread the bacteria on an LB agar plate with appropriate antibiotic and incubate at 37 °C overnight.
2. Inoculate a culture containing 150 mL of LB medium and appropriate antibiotic with a single colony. Shake at 250 rpm and 37 °C overnight.
3. Inoculate the desired volume of LB medium with antibiotic for overexpression. Shake at 250 rpm and 37 °C until $OD_{600\ nm}$ reaches 1.0–1.2 as measured using a spectrophotometer equipped with a turbidity cuvette holder (*see* **Note 3**).
4. Induce NC1 expression by adding IPTG and δ-ALA, both to a final concentration of 1 mM (*see* **Note 4**). Continue to shake for 3.5–4 h at the same temperature. Collect the cells by centrifuging at 5,000 × *g* at 4 °C for 15 min. The pellets should have a noticeable brown color.
5. Store the pellets at –80 °C.

3.2 Purification of NC1

1. Completely resuspend cell pellet in ice-cold DGCR8 lysis buffer (40 mL per L of culture).
2. Sonicate the cell suspension using the ultrasonic processor at 80 % power, 1-s on and 1-s off, for a total of 7–8 min. To avoid overheating the lysate, a 30-s break is taken after each minute of sonication and the container is kept on ice at all times.
3. Centrifuge the lysate at 45,000 × *g* for 30 min at 4 °C.
4. Load the supernatant onto a HiTrap SP column equilibrated with DGCR8 buffer *A*. Elute using a linear gradient of DGCR8 buffers *A* and *B*. The protein elutes at around 300 mM NaCl

(10 % DGCR8 buffer *B*). The fractions containing heme-bound NC1 have a yellowish-brown color. Analyze the purity of the fractions using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

5. If the peak fractions are not >90 % pure, repeat the ion-exchange chromatography step. Pool the fractions containing relatively pure NC1. Dilute it 1:1 (v:v) with DGCR8 buffer *A* to reduce the salt concentration. Repeat **step 4**. The protein may be stored at 4 °C overnight if desired.
6. Equilibrate the Superdex 200 column in SEC buffer (roughly 50 mL). Concentrate the fractions from the last ion-exchange chromatography step down to ~550 μL using a centrifugal concentrator. Filter the concentrated NC1 solution through a membrane with 0.2 μm pores, and load the filtrate onto the column. Collect 0.5 mL fractions. Heme-bound NC1 dimer elutes at around 12.5 mL (*see* **Note 5**).
7. Determine the NC1 protein concentration using UV-visible absorption spectroscopy. Blank the spectrophotometer with SEC buffer. Scan between 240 and 700 nm. An absorption peak at 280 nm indicates that bacterial nucleic acids have been successfully removed from the protein. NC1 dimer concentration = $A_{280\ nm}/\varepsilon_{280\ nm}$. Based on the amino acid sequence, $\varepsilon_{280\ nm}$ is estimated to be 94.5 $mM^{-1}\ cm^{-1}$ ($\varepsilon_{280\ nm,apo}$) [35]. This value has been used in all our previous publications. However, our recent unpublished measurements using microBCA assay indicate $\varepsilon_{280\ nm} \approx 130\ mM^{-1}\ cm^{-1}$ (*see* **Note 6**).
8. Calculate the $A_{450\ nm}/A_{280\ nm}$ ratio. If majority (>60 %) of the NC1 protein is occupied by heme, $A_{450\ nm}/A_{280\ nm}$ should be between 0.40 and 0.53 (the higher the better). NC1 preparations with lower $A_{450\ nm}/A_{280\ nm}$ ratios are less active (*see* **Notes 7** and **8**).
9. The Fe(III) heme-bound NC1 protein may be stored at 4 °C, with protection from light. Because this protein gradually loses pri-miRNA processing activity for reasons not well understood, we typically use it within a couple of days from the completion of purification.

3.3 Expression and Purification of Recombinant Homo Sapiens His_6-$Drosha^{390–1374}$

1. His_6-$Drosha^{390–1374}$ is expressed in Sf9 insect cells using a baculovirus system, following Invitrogen's standard protocols. The cell pellets are stored in −80 °C freezer until purification.
2. Resuspend a pellet from 50 mL of insect cell culture in 30 mL of ice-cold Drosha lysis buffer. Sonicate at 50 % power, 1-s on and 1-s off, for a total of 4 min. To avoid overheating the lysate, a 30-s break is taken after each minute of sonication and the container is kept on ice throughout the sonication procedure.
3. Centrifuge the lysate at 45,000 × *g* for 30 min at 4 °C.

4. Load the supernatant onto a Ni Sepharose High Performance column. Wash the column extensively using the Drosha wash buffer, and elute the His_6-$Drosha^{390–1374}$ protein in the Drosha elution buffer.
5. Dialyze the purified His_6-$Drosha^{390–1374}$ against the Drosha storage/reaction buffer.
6. Aliquot in 10 μL per tube, freeze in liquid nitrogen or dry ice–ethanol mix, and store in −80 °C freezer.

3.4 Transcription and Purification of pri-miRNAs Uniformly Labeled with ^{32}P

1. Set up the transcription reaction by adding the following:

   10 μL H_2O.

   2 μL 10× transcription buffer.

   2 μL 10× NTP mix.

   2 μL DNA template.

   2 μL [α-^{32}P] UTP.

   2 μL T7 RNA polymerase (always add last).

   Incubate the transcription reaction at 37 °C for 2–3 h.
2. Add 20 μL 2× RNA loading dye to the reaction.
3. Pour a denaturing 15 % polyacrylamide gel (1× TBE, 7 M urea). Assemble the gel sandwich. Induce polymerization of the 50 mL gel mix by adding 50 μL TEMED and 500 μL 10 % APS. Pour the gel, insert the comb, and let stand at room temperature for 1 h. Mount the gel on the electrophoresis apparatus. Pre-run the gel in 1× TBE at a constant power of 12 W for 20 min.
4. Load the transcription onto the gel. Run the gel at 12 W until the bromophenol blue reaches the bottom of the gel. Disassemble gel sandwich, and leave the gel attached to one glass plate.
5. Cover the gel with a plastic wrap. Expose the gel to an autoradiography film. Excise out the band. Expose the gel to another film to confirm the excision.
6. Crush and soak the gel piece in 1× TEN buffer at 4 °C for at least 10 h.
7. Precipitate the RNA by adding 3 volumes of ethanol and 0.1 volume of 3 M sodium acetate pH 5.2. Resuspend the RNA in H_2O.
8. Determine radioactivity using scintillation counting. Dilute the RNA to ~10,000 cpm/μL in water prior to the processing assay.

3.5 pri-miRNA Processing Assay

1. Set up a 10-μL processing reaction by mixing the following (in the order shown):

4.5 μL Drosha storage/reaction buffer.

2 μL recombinant His6-Drosha$^{390–1374}$ (~2 ng/μL).

1 μL Fe(III) heme-bound NC1 (10× stock).

0.5 μL RNaseOUT (40 U/μL).

1 μL 64 mM $MgCl_2$.

1 μL pri-miRNA (~10,000 cpm/μL).

For Drosha-only control, the NC1 stock solution should be replaced by SEC buffer. For NC1-only control, Drosha storage/reaction buffer should be used instead of the recombinant Drosha protein (*see* **Note 9**).

2. Incubate at 37 °C for 45 min. The reactions generally do not proceed further after 1 h. Add 10 μL 2× RNA loading dye to stop the reaction.
3. Analyze the reactions using a denaturing 15 % polyacrylamide gel (1× TBE, 7 M urea). Follow **steps 3** and **4** in Subheading 3.4, Transcription and purification of pri-miRNAs.
4. Adhere the disassembled gel on the hydrophobic surface of a Gelbond film (*see* **Note 10**). Cover the other side of the gel with a filter paper. Dry the gel using a gel dryer coupled to a cold trap and a vacuum pump.
5. Expose the gel to a storage phosphor screen overnight.
6. Scan in the image using a phosphorimager. An example image is shown in Fig. 3.
7. Quantify the total intensities of substrate and product bands using an image analysis program. Background intensities are subtracted. To calculate the fraction of pri-miRNA processed, the signals from the pre-miRNAs (*see* **Note 11**) are first converted to that of its corresponding pri-miRNA by multiplying the ratio of U residues in pri-miRNA and pre-miRNA, since the pri-miRNAs were uniformly labeled using [α-^{32}P] UTP. For example, the ratio is 42/16 = 2.625 for the 150-nt pri-miR-30a fragment as we used previously [22]. The signal of pri-miRNA processed is then divided by the amount of starting substrate.

4 Notes

1. The reducing reagent DTT (or β-mercaptoethanol) is important for keeping DGCR8 active. Make sure that your DTT stock solution is in a fully reduced state. Solutions of reduced DTT should have minimal absorbance above 250 nm, while oxidized DTT has an absorbance peak at 283 nm with an extinction coefficient of 273 M^{-1} cm^{-1} [36]. We store our DTT stock solution (1 M) in −20 °C in aliquots and avoid repeated freeze and thaw.

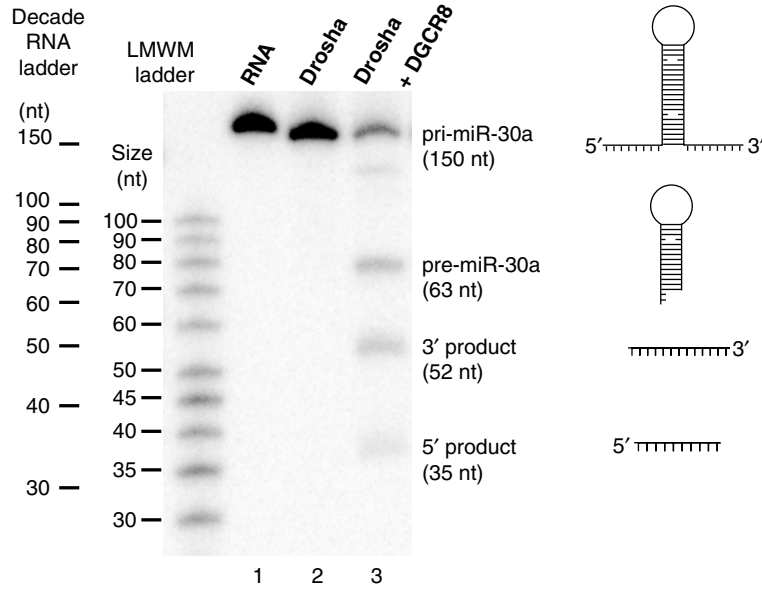

Fig. 3 Example of a pri-miRNA processing assay. Uniformly labeled pri-miR-30a was incubated with 4 nM His_6-$Drosha^{390–1374}$, either alone (*lane 2*) or with 50 nM Fe(III) heme-bound NC1 dimer (*lane 3*), at 37 °C for 45 min. The reactions were analyzed using a 7 M urea, 15 % polyacrylamide gel. LMWM: low molecular weight marker

2. The TEV cleavage site in His_6-$Drosha^{390–1374}$ allows the His_6-tag to be cleaved off using the TEV protease if desired but is not used in the protocol presented here.
3. Measuring $OD_{600\,nm}$ of cell cultures using a spectrophotometer with a turbidity cuvette holder is more accurate than without, because most of the scattered light is blocked by the turbidity cuvette holder. The absolute values of $OD_{600\,nm}$ depend on the instrument used. The bacterial cell density we use in NC1 expression is similar to that commonly recommended for protein expression.
4. δ-ALA is a key heme biosynthesis intermediate. In the absence of δ-ALA, NC1 is expressed as a mixture of heme-bound dimer and heme-free "monomer" [4]. Addition of δ-ALA increases the yield of NC1 expression and improves the heme content of NC1 to the extent that often little or no heme-free "monomer" is observed.
5. Presence of 1 mM DTT or 10 mM β-mercaptoethanol in the SEC buffer helps remove the residual amount of nucleic acids bound to NC1 during size exclusion chromatography using the Superdex 200 column. Under this condition, the free nucleic acids elute at >20 mL, a volume too large for

macromolecules. There seems to be an unusual interaction between nucleic acids and the resin in the presence of thiol-containing reducing reagents.

6. Heme absorbs at 280 nm. Thus, it is not surprising that $\varepsilon_{280\ \mathrm{nm}}$ of the Fe(III) heme–NC1 complex ($\varepsilon_{280\ \mathrm{nm,holo}}$) is higher than that of the protein alone calculated from the amino acid sequence ($\varepsilon_{280\ \mathrm{nm,apo}}$).
7. The extinction coefficient for the 450 nm peak of NC1 is 74 $\mathrm{mM^{-1}\ cm^{-1}}$ [30]. This value was determined using the pyridine hemochromagen assay [37] and should be used instead of the previously reported value (58 $\mathrm{mM^{-1}\ cm^{-1}}$) [4].
8. The heme occupancy (O_{heme}) may be calculated using the following equation:

$$O_{heme} = \frac{\varepsilon_{280,apo} \times \frac{A_{450}}{A_{280}}}{\varepsilon_{450} - \left(\varepsilon_{280,holo} - \varepsilon_{280,apo}\right) \times \frac{A_{450}}{A_{280}}},$$

where the $\varepsilon_{450\ \mathrm{nm}}$ of NC1 is 74 $\mathrm{mM^{-1}\ cm^{-1}}$ [30].

9. The above reaction can also be set up anaerobically using an anaerobic chamber to prepare the protein sample and a gastight syringe to transfer the pri-miRNA to the sample [6]. To do kinetic assay, increase the above volumes and take aliquots at desired intervals.
10. Polyacrylamide gels ≥15 % do not stick strongly to filter paper typically used in gel drying protocols but adhere to the hydrophobic surface of the Gelbond film. Note that this surface is opposite to the treated hydrophilic side that is designed to cross-link with acrylamide.
11. There is a low level of nonspecific nuclease activity associated with the $\mathrm{His_6}$-$\mathrm{Drosha^{390–1374}}$ protein, which may originate from either Drosha itself or residual impurities present in the Drosha preparation. The pre-miRNA is usually the most stable among the three specific Drosha cleavage products and thus is used for quantification.

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