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A fluorescence probe for assaying micro RNA maturation

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Abstract—A class of small RNAs known as micro-RNAs (miRNAs) has been shown to play an important role in development and cellular regulation in eukaryotes. Recent evidence also associates aberrant expression of various miRNAs with multiple diseases in humans including many types of cancer. An up- or downregulation of certain miRNAs may play a significant role in the course of these diseases. We were interested in whether binding of small molecules to the inactive miRNA precursors would block their cleavage into the active miRNAs by the enzyme Dicer. The inhibition of miRNA maturation might provide a new rationale for future therapeutic strategies. We have developed a fluorescence beacon with which to study the maturation of miRNAs. Details are provided of a homogeneous assay for detecting potential inhibitors of miRNA maturation in a high-throughput format.

1. Introduction

In recent years there has been a tremendous amount of research done into understanding the role that micro-RNAs (miRNAs) play for both regulation and development in eukaryotes.[1] Moreover, an ever increasing amount of data implicates an interplay of these small double-stranded RNAs in disease processes.[2]

miRNAs are double-stranded RNAs about 21–25 nucleotides long that regulate protein expression. The miRNA, together with various proteins, forms what is known as a micro-RNA–protein complex (miRNP complex), which through non-perfect hybridization with the target gene leads to blockage of protein translation. Some miRNAs do hybridize perfectly with their respective targets, thus leading to cleavage of the target sequence with the same result. miRNAs are formed in the cytoplasm from preliminary miRNA molecules (pre-miRNA) of about 70–80 nucleotides in length, which have a hairpin structure containing a 5′-phosphate and 3′-overhang of two nucleotides. Dicer cleaves at both sides of the pre-miRNA helical stem about 21 nucleotides from the open helical end to form the final miRNAs which also contain 5′-monophosphates and the 3′-overhangs.[3]

Hundreds of miRNAs are now known in each of various species and it is believed that they make up at least 1% of the human genome.[4] Furthermore, it is estimated that up to 30% of all human mRNAs can be targeted by miRNAs.[5] The obvious role of miRNAs in gene regulation also suggests that these molecules are involved in disease development.[6–8] Recently, in fact, numerous cases have been reported in which disease states correlate with altered miRNA expression profiles.[9–11] Early research on miRNAs has shown that, for example, mitosis and cell differentiation are controlled by these regulatory nucleic acids. This finding is corroborated by the fact that changes in miRNA expression patterns, in particular, have been found in various cancers. In some cases, a causal connection between miRNA expression and tumor development already has been experimentally proven.[8]

The vast amount of data pointing at a connection between miRNA expression and disease development strongly suggests the need for the development of novel therapeutic strategies directed at the manipulation of miRNA expression. We reasoned that specific binders of pre-miRNAs might interfere with the miRNA formation process. Such molecules might be able to reduce the cellular concentration of pathogenic miRNAs. To this end we have developed a pre-miRNA fluorescence beacon for which potential binders can be screened in a high-throughput format.[12]

The fluorescent probe was designed by placing a 5′-fluorophore and a 3′-fluorescence quencher at the ends of a

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pre-miRNA hairpin structure. In the natural conformation the fluorophore would be in close proximity to the quencher and no fluorescence should be seen. When Dicer is present, the beacon would be cleaved, setting free the fluorophore and quencher, and thus leading to an increase in fluorescence. In the presence of an RNA binder Dicer would be blocked and a lowered or lack of fluorescence should be observed.

2. Results and discussion

2.1. Fluorescent beacon synthesis

A 72-nucleotide long sequence based on pre-let-7 RNA from *Drosophila melanogaster* was chosen as the basis for the fluorescent beacon, as the respective let-7 miRNA was the first shown to be cleaved from a longer RNA strand by Dicer.[13] The fluorophore fluorescein (FAM) was used at the 5′-end with the fluorescence quencher Dabcyl at the 3′-end (Fig. 2). The blunt end of the pre-let-7 hairpin places the fluorophore and quencher nearly opposite one another, thus leading to quenching of the FAM fluorescence in the hairpin structure. Moreover, such a hairpin RNA also allows for an improved release of the FAM group because of the way Dicer cleaves such a structure (Fig. 1).

Whereas a pre-miRNA with a 3′-overhang of ~2 nucleotides is cleaved only once, approximately 21–25 bases from the open end,[3] with a blunt end hairpin a longer section is first cleaved from the hairpin and from this the typical 21–25-mers are then cleaved.[14] A blunt end structure results in a total of four phosphodiester bonds being hydrolyzed in contrast to a natural pre-miRNA. According to this pattern our beacon is cleaved into a short terminal FAM/Dabcyl fragment, a middle section containing the ~22-mers, and the hairpin section itself (Fig. 1). This sets free a significantly shorter length fragment containing the FAM group as would be the case with a structure more closely resembling a natural pre-miRNA, thus guaranteeing an effective fluorescent signal upon Dicer-mediated cleavage.

The beacon was formed by ligating a 5′-FAM labeled 36-mer together with a 3′-Dabcyl-labeled 36-mer also containing a 5′-phosphate (Fig. 2). Ligation using T4 RNA ligase allowed a high yield as seen by PAGE (Fig. 3). Native gel PAGE shows no significant fluorescence of the product strand (gel b, lane 4) without stain. In addition, the 3′-Dabcyl 36-mer shows no fluorescence as expected (gel b, lane 2), but even a simple mixture of the two 36-mers leads to some quenching of the FAM fluorescence (gels a/b, lane 3). Upon staining with SYBR Green the product strand can be seen, however the 3′-Dabcyl 36-mer is only slightly visualizable owing to its effective fluorescence quenching.

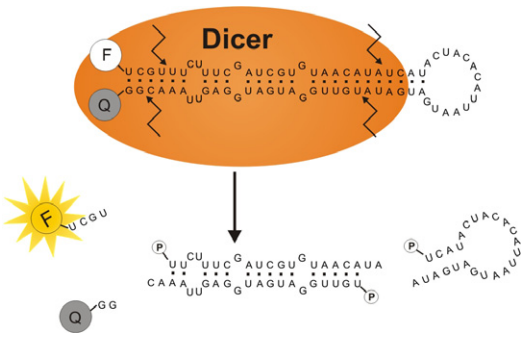

Figure 1. Cleavage of a blunt end pre-miRNA probe by Dicer results in fluorescence emission.

The product was initially phenol extracted to remove protein. The lipophilic FAM/Dabcyl groups in the beacon then allowed easy purification via reverse-phase HPLC (Fig. 4). The yield of the ligation reaction based on integration of the product peak in the HPLC chromatogram gave 74% (corrected for solvent front absorbance). MALDI-TOF analysis confirmed the purity of the product beacon (Fig. 4). The pure fractions were combined and lyophilized. The beacon was stored in 1 mM ammonium citrate, pH 6.4, until further use.

Melting curve analysis of the purified beacon showed sigmoidal behavior as evidence for the hybridized hairpin structure. The sample was first taken up in buffer and renatured before measuring. The first derivative gave a T_M of 59 °C. This high value is expected for such a long RNA hairpin structure. Thus, our blunt end hairpin design allowing the release of a shorter FAM-fragment is critical.

2.2. Cleavage with Dicer

The next step was to see if Dicer could actually cleave our fluorescence beacon. Using commercial recombinant Dicer (Stratagene or Genlantis) a Dicer-mediated fluorescence increase was observed over the course of 18 h (Fig. 4). The NaCl concentration (250 mM) was according to the manufacturer's conditions, except that

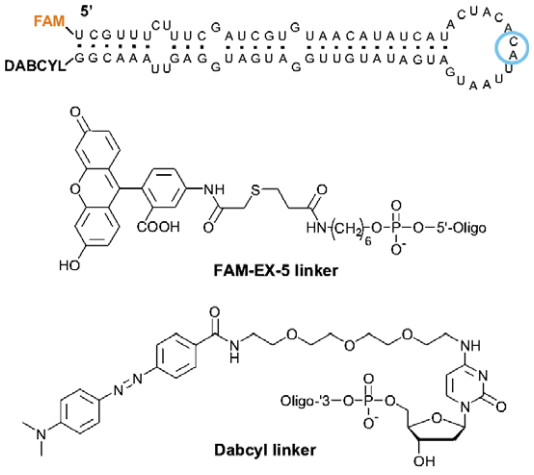

Figure 2. Fluorescence beacon shown in its hairpin conformation[15] with ligation point marked in blue together with structures of 5′-fluorescein modification (FAM-EX-5 linker) and 3′-Dabcyl linker (IBA GmbH, Germany).

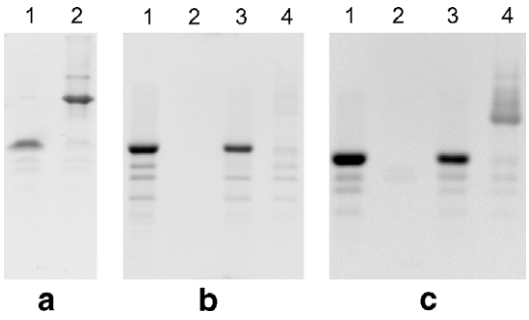

Figure 3. (a) Denaturing PAGE of ligation reaction, (lane 1) fragments mixed, (lane 2) ligation product. (b) Native PAGE of ligation without staining, lane 1, FAM 36-mer; lane 2, Dabcyl 36-mer; lane 3, fragments mixed; lane 4, ligation product. (c) Same as in (b) except stained with SYBR Green. Twenty percent gels were used.

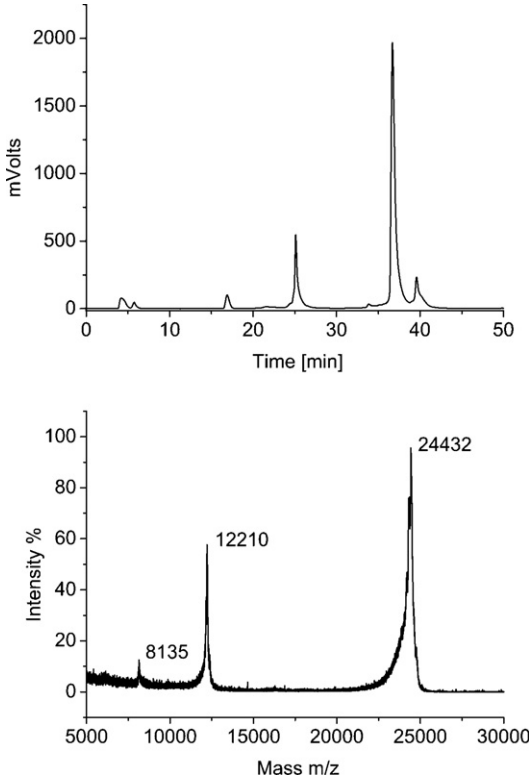

Figure 4. (Top) HPLC chromatogram from purification of beacon, t_R = 37 min. (Bottom) MALDI-TOF spectrum of beacon, calcd: $[M-H]^-/z$ 24,411; $[M-2H]^{2-}/2z$ 12,205; $[M-3H]^{3-}/3z$ 8136. Found: $[M-H]^-/z$ 24,432; $[M-2H]^{2-}/2z$ 12,210; $[M-3H]^{3-}/3z$ 8135.

1 mM DTT was also added. We observed a slow rate of cleavage, which was in agreement with the manufacturer's specifications. The recombinant Dicer enzyme is known though to be quite inefficient.[3,16]

In the cell Dicer interacts, however, with various proteins such as transactivating response binding protein (TRBP), which increases the Dicer-mediated cleavage in vitro and is required in vivo for proper functioning of the enzyme.[17] Incubation of the beacon in the presence of cell lysate from human embryonic kidney cells (HEK293) leads to a much faster and overall greater fluorescence signal (Fig. 6). Compared to the initial study (Fig. 5) we were able to use a significantly lower concentration of the beacon (20 nM compared with 700 nM). Despite the lowered concentration of beacon peak fluorescence was nearly reached after only 3 h with a 6-fold increase in fluorescence compared to the control.

Because of concerns over possible RNAse in the cell lysate we also tested the effect of RNAse inhibitor on the reaction. As well, in order to develop an assay suitable for high-throughput measurements we now shifted to measurements with a plate reader using 96-well plates.

The addition of RNAse inhibitor to the cleavage reaction led to a virtually identical fluorescence increase as compared to the reaction without RNAse inhibitor (Fig. 7). Thus, in further experiments using cell lysate as the Dicer source, we were able to forgo the use of RNAse inhibitor.

We wanted to use recombinant enzyme, however, since this would minimize the interference of other proteins and—probably more importantly—cellular nucleic acids. We also wished to increase its cleavage efficiency. We determined that addition of 1 mM DTT led to a

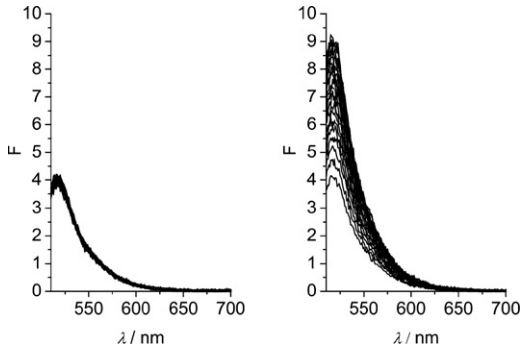

Figure 5. Incubation of 700 nM beacon with heat-denatured recombinant Dicer (left) compared to that with recombinant Dicer (right). Conditions: 25 U Dicer, 20 mM Tris–HCl, pH 7.4, 250 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT in 1 mL. Measurements were taken every hour for 18 h.

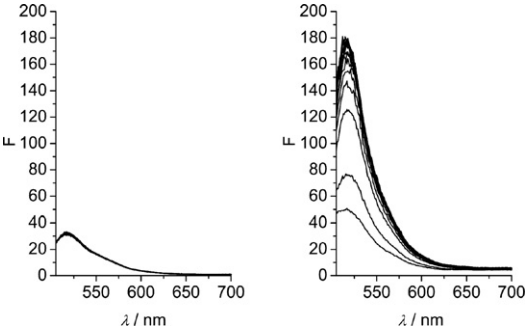

Figure 6. Fluorescence increase upon incubation of 20 nM beacon with 10% heat-denatured HEK293 lysate (left) compared to that with 10% HEK293 lysate (right). Further conditions: 20 mM Tris–HCl, pH 7.4, 75 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT in 1.1 mL. Measurements were taken every 30 min for 6 h.

faster and greater fluorescence increase. In addition, when we lowered the sodium chloride concentration from 250 to 150 mM we also observed a much larger fluorescence increase due to Dicer (Fig. 8). Interesting to note is that at 250 mM NaCl (no DTT) there is, in fact, no observable fluorescence increase. This result supports our earlier cleavage experiments using the recombinant Dicer in cases where no DTT was present, the manufacturer's buffer was used, or where the NaCl concentration was very high. Under such conditions cleavage activity was observed only when larger amounts of the enzyme were used (Fig. 5).

Further reduction of the NaCl concentration down to 12 mM showed the greatest cleavage activity of commercially available recombinant Dicer (Fig. 9). This is in agreement with published data on recombinant Dicer.[16,18] Under the improved assay conditions, we were able to use as little as 10 nM fluorescence beacon. Instead of incubation times of up to 18 h, the fluorescence reached a plateau after only one hour (Fig. 9).

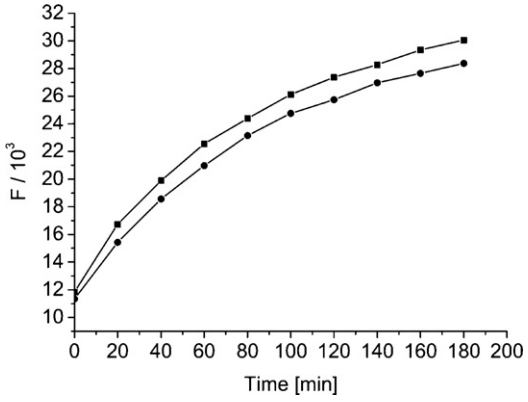

Figure 7. Fluorescence increase upon incubation of 50 nM beacon with (●) and without (■) RNAse inhibitor in the presence of 10% cell lysate. Further conditions: 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$ in 100 μL in 96-well plate.

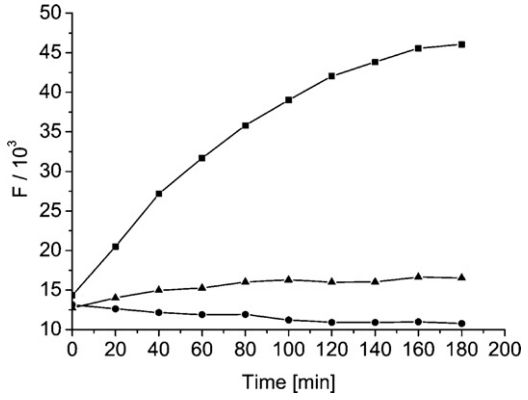

Figure 8. Fluorescence increase upon incubation of 50 nM beacon with 1 U recombinant Dicer in the presence of 250 mM NaCl (●), 150 mM NaCl (▲) or 150 mM NaCl + 1 mM DTT (■). Further conditions: 20 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$ in 100 μL in 96-well plate.

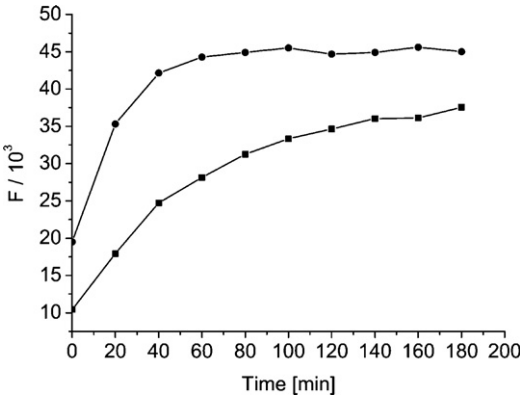

Figure 9. Fluorescence increase upon incubation of 10 nM beacon with 0.25 U recombinant Dicer in the presence of 75 mM NaCl (●) or 12 mM NaCl (■). Further conditions: 10 nM beacon, 20 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 1 mM DTT in 40 μL in 384-well plate.

3. Detection of cleavage products

We wanted to verify that the Dicer cleavage of our fluorescence beacon was actually occurring as expected.[14] We compared the cleavage to that of the unlabeled pre-let-7 hairpin, obtained through in vitro transcription. This was then dephosphorylated (alkaline phosphatase) and subsequently rephosphorylated in the presence of ATP and polynucleotide kinase in order to obtain a 5′-monophosphate. Complete turnover at each step was verified using MALDI-TOF as described. We performed a cleavage of both our fluorescence beacon as well as the unlabeled in vitro transcript under identical conditions using recombinant Dicer and visualized the cleavage products using denaturing PAGE (Fig. 10).

In both cases bands can be seen, which were determined by MALDI-TOF analysis to be in the range of 21–25 nucleotides in length. Excision and elution of the respec-

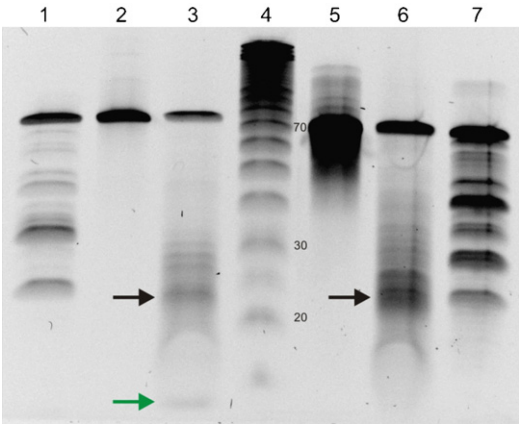

Figure 10. Fifteen percent denaturing PAGE gel stained with SYBR Gold showing Dicer cleavages of both beacon (lane 3) and the homologous in vitro transcript (lane 6) with controls (lanes 2 and 5, respectively). The expected bands corresponding to ~22-mers can be seen (black arrows). The green arrow points out the short 5′-FAM fragment. Lanes 1 and 7 show T1 RNAse digests of the beacon and transcript, respectively. Lane 4 is a size marker with pertinent sizes labeled.

tive bands from the gel using the 'crush and soak' method and subsequent alcohol precipitation allowed for MALDI-TOF analysis of the fragments. In addition, a much faster running band at the bottom, which self-fluoresced, belongs to the short 5′-FAM fragment. Upon staining with SYBR Gold, however, the shorter fragments become less intense, most likely due to their elution from the gel over time.

4. Testing of RNA binders

In order to test if our probe is suitable for identifying inhibitors of miRNA maturation, we used the unspecific binder kanamycin A to show the proof of principle.[19] Indeed, at a concentration of 100 μM, kanamycin A showed approximately 73% inhibition after 1 h (Fig. 11).

We also observed that when using the cell lysate as the Dicer source, inhibition by kanamycin A was substantially lower. This could be explained by unspecific binding of kanamycin A to cellular nucleic acids in the cell lysate, thus reducing its effective concentration.

5. Conclusions

Regulation of disease processes at the RNA level is becoming more promising with the discovery of miRNAs. Ligands which specifically bind pre-miRNAs could offer a therapeutic route for such diseases as cancer where overexpression of the respective miRNA is shown to be a contributing factor in pathogenesis.

With our pre-let-7 fluorescence beacon we have established the first homogeneous fluorescence assay for detecting potential inhibitors of the miRNA maturation process. Our fluorescein-based beacon provides for a particularly sensitive fluorescence signal, allowing the detection of potential inhibitors using only 10 nM beacon.

We have also been able to optimize the assay by improving the activity of commercial Dicer through a simple change of buffer salt concentrations, thus drastically improving Dicer's cleavage efficiency. This allows easy detection in less than 1 h using as little as only 0.25 U/well commercial recombinant Dicer. The efficiency and the associated low cost (roughly one Euro cent per well based on the beacon) would allow the screening of large libraries of potential pre-miRNA binders.

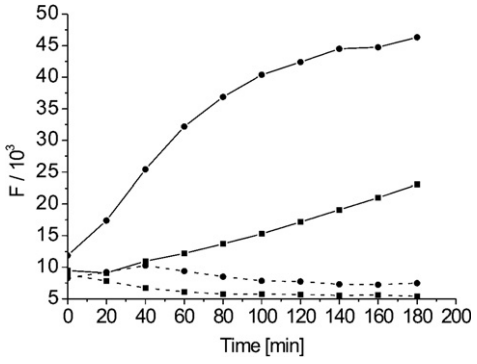

Figure 11. Fluorescence increase upon incubation of 0.5 U recombinant Dicer with 20 nM beacon alone (—●—) or in the presence of 100 μM kanamycin A (—■—). Controls contain heat denatured recombinant Dicer with (- - -■- - -) or without (- - -●- - -) 100 μM kanamycin A. Further conditions: 20 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 1 mM DTT in 40 μL in a 384-well plate.

Moreover, cell lysate may be used as the Dicer source, thus allowing investigation of a potential inhibitor's effects under more realistic conditions. A ligand that is highly specific for a particular pre-miRNA would lead to a decreased fluorescence signal despite the presence of other nucleic acids. A general RNA binder would also tend to bind cellular nucleic acids in the cell lysate, thus leading to a lowering of its effective concentration in the assay and a greater fluorescence increase.

The object of future work needs to address the issue of selectivity with pre-miRNA binders. A possible screen for specific pre-miRNA binders could be established by designing a second pre-miRNA beacon carrying a fluorescence emitter other than the FAM group. This would not only allow for simultaneously measuring the cleavage of a second pre-miRNA but also provide information about potential inhibitiors of the enzyme Dicer itself.

Another aspect that would make such molecules of real therapeutic interest would be their uptake by the organism. On this point small organic molecules might provide a particular advantage compared to antisense technologies[20] because of an increased uptake by the organism as well as lower manufacturing costs.

6. Experimental

6.1. General

All reagents used were of highest quality available and, when possible, certified RNAse free. Water was purified with a Milli-Q® Ultrapure Water Purification System (Millipore Corp.). All buffers were additionally sterile filtered through 0.22 μM filters.

MALDI-TOF measurements were performed on a Voyager-DE Pro Biospectrometry Workstation from PerSeptive Biosystems (Foster City, USA). Measurements were done in linear and negative ion mode. Masses measured are the average masses. A matrix THAP-citrate in a ratio of 2:1 v/v was made from 0.3 M THAP (2,4,6-trihydroxyacetophenone) in ethanol and 0.1 M diammonium citrate (pH 6.4) in water.

Reverse-phase HPLC (RP-HPLC) was performed on a Gilson 321 HPLC using a 'Polaris' column from Varian Inc. (5 μm, 200Å, 250 mm × 4.6 mm) with UV detection at 260 nm. Columns were heated at 50 °C. Acetonitrile gradients (eluent A) were used in 0.1 M TEAA (eluent B) with a flow rate of 1 mL/min. Extinction coefficients for the RNA were calculated using the nearest neighbor method.[21] The extinction coefficient for the beacon was calculated as the sum of the unmodified RNA together

with the extinction coefficient for the FAM (ε_{260} = 21,000) and Dabcyl (ε_{260} = 7000) moieties. Hypochromicity effects due to hairpin formation were not taken into account.

Fluorescence measurements were all done twice on either a Varian Cary Eclipse fluorescence spectrophotometer or a BMG Labtech Fluorostar Optima plate reader and the average values given. For optical analyses a Varian Cary 100 Bio-UV/visible spectrophotometer was used with a path length of 1 cm.

PAGE analyses were done either with native or denaturing (8 M urea) gels using a 15% or 20 % running and 4% stacking gels (bis:acrylamide, 1:19). A constant voltage of 250 V in TBE buffer, pH 8.3, was applied. Samples were loaded with formamide loading buffer containing 95% formamide, 18 mM EDTA, and 0.1% bromophenol blue at least at a 1:1 ratio and denatured at 95 °C for 5 min before loading. Gels were viewed under UV light and photographed using a CCD camera. Either SYBR Green or SYBR Gold (Invitrogen, 1:10,000 dilution in 1× TBE buffer) was used for staining gels.

Recombinant human Dicer was purchased either from Stratagene or Gene Therapy Systems (Genlantis). RNAse inhibitor (RNAsin) was supplied by Promega.

6.2. Beacon ligation

The 36-mers with 5′- and 3′-modifications for producing the beacon were purchased from IBA GmbH (Göttingen, Germany) with double HPLC purification. The 5′-FAM-EX-5-labeled 36-mer (5′-FAM-EX-GGCAA AUUGAGGUAGUAGGUUGUAUAGUAGUAAU UA) (100 μL, 10 nmol) was incubated together with the 3′-Dabcyl- and 5′-phosphate-labeled 36-mer (5′-PO_3-CACAUCAUACUAUACAAUGUGCUAGCUUUC UUUGCU-DABCYL) (100 μL, 10 nmol), 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 200 U RNAsin, and 200 U T4 Ligase (New England Biolabs) at pH 7.8 in a total volume of 239 μL for 18 h at 37 °C. The reaction was phenol extracted (phenol–chloroform–isoamyl alcohol, 25:24:1) and the raw product precipitated with Na-acetate in isopropanol and then purified by RP-HPLC using a gradient of 3% B for 5 min and then from 3% to 40% B over 40 min, t_R = 37 min. Analysis was done by MALDI-TOF. Calcd: $[M-H]^-/z$ 24411, $[M-2H]^{2-}/2z$ 12205, $[M-3H]^{3-}/3z$ 8136. Found: $[M-H]^-/z$ 24432, $[M-2H]^{2-}/2z$ 12210, $[M-3H]^{3-}/3z$ 8135). Pure fractions were lyophilized. The beacon was then stored in 1 mM ammonium citrate, pH 6.4. Prior to use in Dicer experiments the beacon was taken up in buffer containing 40 mM NaCl, 20 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$ and 1 mM DTT, heated for 2 min at 95 °C, and then allowed to renature by cooling at room temperature.

6.3. T_M determination for beacon

The T_M for the beacon was measured in buffer containing 250 mM NaCl, 20 mM Tris–HCl, pH 7.4, and 2.5 $MgCl_2$. Heating and cooling rates of 1 °C/min were used. The sample was first renatured by heating to 85 °C and then cooling to 5 °C. A T_M = 59 °C was then calculated as the average of three measurements from the maximums of the first derivatives.

6.4. In vitro transcription

A Promega T7 RiboMAX Express in vitro transcription kit was used. DNA was purchased with double HPLC purification from Proligo (Germany). The DNA template with underlined T7-promoter sequence is as follows:

5′-AGCAAAGAAAGCTAGCACATTGTATAGTAT
GATGTGTAATTACTACTATACAACCTACTACC
TCAATTTGCCTATAGTGAGTCGTATTA

The following complementary T7-primer was used:

5′-GGTAATACGACTCACTATAG.

After transcription according to the manufacturer the RNA was dephosphorylated with alkaline phosphatase and rephosphorylated with polynucleotide kinase (both from New England Biolabs). The product was purified by phenol extraction followed by RP-HPLC as described above using a gradient of 0% B for 5 min and from 0% to 20% B in 40 min, t_R = 33 min. Analysis by MALDI-TOF gave calcd: $[M-H]^-/z$ 23121, $[M-2H]^{2-}/2z$ 11563, $[M-3H]^{3-}/3z$ 7703. Found: $[M-H]^-/z$ 23135, $[M-2H]^{2-}/2z$ 11564, $[M-3H]^{3-}/3z$ 7710. Pure fractions containing some $n+1$ product were lyophilized. The RNA was then stored and renatured before use as described for the beacon above.

6.5. Cell lysate

Human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen). The cells were trypsinated, collected, and centrifuged at 2000*g* and 4 °C for 5 min. The supernatant was discarded and the pellet washed once with phosphate-buffered saline and again centrifuged at 2000*g* and 4 °C for 5 min. The supernatant was discarded and the pellet taken up in buffer containing 20 mM Tris–HCl, pH 7.4, 75 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 10% glycerol, and Roche protease inhibitor cocktail. The cells were lysed by ultrasound and centrifuged once at 4300*g* and 4 °C for 10 min. The supernatant was then transferred to a fresh reaction vial and centrifuged again at 12,100*g* for 5–10 min at room temperature. The supernatant was transferred once again to a fresh reaction vial and stored at −20 °C until further use. Total protein concentration was determined by the Bradford assay[22] and contained between 100 and 250 μg/mL.

6.6. Assay conditions

The Dicer assay was performed in 96- or 384-well plates. For 384-well plates a final volume of 40 μL was used. An optimized 40 μL reaction contained 10 nM beacon, 20 mM Tris–HCl, pH 7.4, 12mM NaCl, 2.5 mM $MgCl_2$, and 1 mM DTT. Reaction mixtures containing kanamy-

cin A were pre-incubated at room temperature for 30 min. Dicer was then added, either 0.25 U commercial recombinant Dicer or 10% HEK293 cell lysate, and the fluorescence increase measured every minute for 4 h.

Acknowledgments

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