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# Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot

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## ABSTRACT

**The northern blot, or RNA gel blot, is a widely used method for the discovery, validation and expression analysis of small regulatory RNA such as small interfering RNA (siRNA), microRNA (miRNA) and piwi-interacting RNA (piRNA). Although it is straightforward and quantitative, the main disadvantage of a northern blot is that it detects such RNA less sensitively than most other approaches. We found that the standard dose of UV used in northern blots was not the most efficient at immobilizing small RNA of 20–40 nt on nylon membranes. However, increasing the dose of UV reduced the detection of miRNA by hybridization in northern blotting experiments. We discovered that using the soluble carbodiimide, EDC, to cross-link RNA to nylon membranes greatly improved the detection of small RNA by hybridization. Compared to standard UV cross-linking procedures, EDC cross-linking provided a 25–50-fold increase in the sensitivity of detection of siRNA from plants and miRNA or piRNA from mammalian cells. All types of hybridization probes tested benefited from the new cross-linking procedure. Cross-linking was dependent on a terminal phosphate and so, should be applicable to other related categories of small RNA.**

## INTRODUCTION

Small regulatory RNAs, which direct the suppression of gene expression in eukaryotes, are now the subject of intensive investigation in many model organisms (1,2). A common feature of all such RNA is that their sizes always fall within a narrow, defined range of between 19 and 31 nt (1–3). For some, including the most prominent classes of siRNA and miRNA, that precise size is a direct result of biosynthesis catalysed by RNAse III-like enzymes known as DICERs (4–6). For others, such as piRNA [review by (3)] and repeat associated short interfering RNA (rasiRNA) repeat associated short interfering RNA (7), the mechanism of synthesis is not yet known. Nonetheless, the defined, discrete size of all of these small regulatory RNA species distinguishes them from catabolic fragments of longer cellular RNA and so, is a valuable identifying feature. Moreover, the precise size (i.e. single nucleotide resolution) of a small regulatory RNA has also been shown in certain cases to be indicative of a specific function (3,8).

As the interest in silencing-related small RNA has grown, there have been several improvements in methodology for their identification, validation and expression profiling (9). A widely used method is 'northern blotting' or 'RNA-gel blotting': denaturing polyacrylamide gel electrophoresis of RNA followed by electrophoretic or capillary transfer of the RNA onto a solid support and then detection by hybridization with a labelled, complementary nucleic acid probe. This procedure is less sensitive than RT-PCR-based approaches and has lower throughput than cDNA cloning, 'deep' sequencing or expression analysis with microarrays. However, northern blotting is quantitative, technically straightforward, relatively inexpensive and is often employed to validate small RNA identified by higher throughput methods. It is the most convincing way to display the size distribution of small RNA and can simultaneously display the levels of mature and precursor forms of a miRNA. Small RNA northern blots using candidate-sequence probes are also useful for the discovery of siRNA because these small RNA appear to lack precise precursors analogous to those of miRNA

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which can be identified *a priori* by computer-based approaches (10). Other RNA such as piRNA and rasiRNA which do not arise from transcription of obvious inverted repeats are similarly difficult to predict and/or validate by *in silico* methods (3,7). Small RNA northern blotting can also be used to determine the amount and size of siRNA produced from transgenic constructs created to knockdown gene expression and so help to monitor and troubleshoot these experiments.

The standard small RNA northern blot method for small RNA (11) is identical in principle to that used successfully for many years to study longer RNA species such as mRNA (12). An important step is the UV-induced cross-linking of the sample RNA to a nylon 6,6 membrane support that increases the retention of the RNA during subsequent immersion of the membrane in hybridization, washing and probe-stripping solutions. UV-cross-linking is believed to take place primarily through the thymine bases of DNA, and, by inference, the uridine residues of RNA. Uncharacterized reactive species produced are thought then to form covalent cross-links with free amine groups at the nylon membrane surface (13,14). Although the exact mechanism is not known, it would seem that cross-linking via the nucleotide bases could compromise subsequent hybridization by consuming the required functional groups, reducing the longest length of uninterrupted sequence or limiting the free movement of the target polynucleotide chain. We hypothesized that any of these factors might be particularly problematic for the detection of small RNA such as siRNA and miRNA where even one cross-linked base—presumably the minimum requirement for retention—could compromise hybridization with complementary nucleic acid probes.

Here we show that UV cross-linking is, to an extent, antagonistic to hybridization of small RNA and improved detection of small RNA cannot be achieved simply by increasing the dose of UV. Instead, the UV step can be omitted and the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) used to cross-link RNA to nylon membranes at least as efficiently. EDC-cross-linking likely proceeds via a 5′ terminal phosphate and results in an immobilized RNA that is much more amenable to detection by hybridization to complementary nucleic acid probes. We show that greatly improved detection of siRNA, miRNA and piRNA is possible with this modification. Given the similarities in end chemistry of most small regulatory RNA, this improvement to small RNA northern blotting should have widespread applications.

## MATERIALS AND METHODS

### Plant growth conditions

*Arabidopsis thaliana* (Columbia) were grown under long-day glasshouse conditions at ~22°C. Flowers were harvested from 5- to 6-week-old plants. Untransformed *Nicotiana benthamiana* and the transgenic line GFP16c/GFPi (15) were grown from seed for 4 weeks at 25°C under 16 h light/8 h dark in a Sanyo Environmental Test chamber.

### Mammalian cell lines, cell culture and tissue

Mouse embryonic stem (ES) and HeLa (ECCAC-Sigma) cells were grown under standard conditions of 5% $CO_2$ at 37°C. Undifferentiated ES cells were grown on gelatinized tissue culture plasticware in high glucose DMEM (GIBCO) supplemented with 20% fetal bovine serum (FBS) (Autogen Bioclear), 0.1 mM non-essential amino acids, 2 mM L-glutamine, 0.06 mM β-mercaptoethanol, 100 U ml$^{-1}$ penicillin, 100 μg ml$^{-1}$ streptomycin, and $10^3$ U ml$^{-1}$of LIF (Chemicon). ES cells were allowed to differentiate spontaneously for 4 days in media containing no LIF.

HeLa cells were grown in EMEM (Sigma) supplemented with 10% FBS (Autogen Bioclear), 0.1 mM non-essential amino acids and 2 mM L-glutamine. All tissue culture reagents were purchased from Invitrogen unless otherwise stated.

Animals were culled using a Schedule 1 method in accordance with the UK Home Office Regulations. Brain, heart, liver and testes tissue were harvested from 4-month-old male rats (Fischer) and 3-month-old male mice (C57BL/6), and mammary tissue was harvested from 3-month-old female mice (BALB/c) and frozen immediately in liquid nitrogen.

### RNA extraction

*Plant*. Tissue was frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle, homogenized in TRI-Reagent® (Sigma) and total RNA extracted according to the manufacturer's instructions with the following additional steps. Samples were extracted with an equal volume of Tris/HCl- buffered phenol/chloroform (Sigma) after chloroform extraction and prior to precipitation with isopropanol. After dissolving the nucleic acid pellets in DEPC-treated water, they were treated with TurboDNAse® (Ambion) in the manufacturer's buffer with ~1 U per 100 μg of RNA at 37°C for 1 h. Samples were then extracted with an equal volume of Tris/HCl-buffered phenol–chloroform (Sigma), precipitated with an equal volume of isopropanol and one-tenth volume of 3 M sodium acetate (pH 6) and re-dissolved in DEPC-treated water.

*Animal*. Cultured mammalian cells were lysed and homogenized directly in TRI-Reagent® (Sigma) and RNA extracted as above with modified manufacturer's instructions. HeLa cell RNA was treated with TurboDNAse® as described above for RNA from plant tissue. Animal tissue frozen in liquid nitrogen was ground to a fine powder, homogenized in TRI-Reagent® (Sigma) and RNA extracted as for HeLa cells.

RNA was dissolved in DEPC-treated water and quantified by measuring the absorbance at 260 nm using a Nanodrop® UV spectrophotometer (Labtech). Deionized formamide (Sigma) was then added to a final concentration of 50% and the RNA stored at −20°C until use.

Dephosphorylation of total RNA involved treatment of the RNA with 5 U of bacterial alkaline phosphatase (Invitrogen) per μg of total RNA at 60°C for 1 h.

Enrichment for low molecular weight RNA was by differential polyethylene glycol (PEG) precipitation as described in ref. (11).

### Electrophoresis and blotting of RNA

All RNA samples were separated by electrophoresis using either 10 or 15% polyacrylamide (19:1) gels cast in 7 M urea and buffered with 20 mM MOPS/NaOH (pH 7) using a Protean II rig (Bio-Rad). Tris-based buffers were avoided as it was anticipated that these might interfere with the EDC-mediated cross-linking step. The electrophoresis buffer was 20 mM MOPS/NaOH (pH 7). RNA markers were $^{32}\gamma$P-end-labelled Decade® RNA markers (Ambion) prepared according to the manufacturer's instructions. After gel electrophoresis, gels were stained with ethidium bromide (EtBr) or SybrGold® (Molecular Probes) and imaged on a FLA-5000 system (Fuji) with Aida Image Analyser software to visualize and record the amount and distribution of the RNA.

For blotting, gels were placed over a sheet of nylon hybridization membrane (Hybond-NX®, Amersham/Pharmacia) that had been pre-wetted in distilled water. This was then sandwiched between pieces of 3MM® Whatman filter paper (three layers on each side), also pre-wetted in distilled water and placed in a 'semidry' electroblotter (SciPlas). Excess liquid and air bubbles were squeezed from the sandwich by rolling the surface with a clean pipette. Electrophoretic transfer of RNA from the gel to the membrane was carried out at 20 V at 4°C for 30–60 min.

### Cross-linking of RNA to Hybond-NX®

*EDC*. Immediately prior to use, a solution of 0.16 M l-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma) was prepared in 0.13 M 1-methylimidazole at pH 8 (pH adjusted with HCl). In each experiment, the prepared volume of this cross-linking solution was just sufficient to saturate a single sheet of 3MM® Whatman filter paper that had been cut slightly larger than the area of the hybridization membrane. For example, to cross-link RNA transferred onto a piece of Hybond-NX® nylon membrane 20 × 16 cm (i.e. 320 $cm^2$), 122.5 µl of 12.5 M 1-methylimidazole was added to 10 ml of DEPC-treated $H_2O$ and adjusted to pH 8 with 1 M HCl. To this, 0.373 g of EDC was added and the volume made up to 12 ml. The membrane was placed on the EDC-saturated 3MM® paper with the side onto which the RNA had been transferred facing up. This was then wrapped in SARAN wrap and incubated at 60°C for between 15 minutes and 2 hours. The membrane was then washed in distilled water to remove residual EDC solution prior to prehybridization. Membranes can be dried and stored at −20°C after removal of residual EDC with no detrimental effects (data not shown).

*UV*. Following transfer, membranes were immediately placed in the cabinet of a commercial UV irradiation device (Stratalinker®, Stratagene, CA, USA) and a prescribed amount of UV energy delivered. In most experiments, this was 0.24 J.

### Probe synthesis

*In vitro* transcription of DNA templates was carried out with 20–40 U of T7 RNA polymerase per reaction and $\alpha$-$^{32}$P UTP (800 Ci·mmol$^{-1}$; 10 mCi·ml$^{-1}$). Probes for TS and GFP siRNA were generated by *in vitro* transcription reactions from plasmid templates [500 µg ml$^{-1}$ of pTSa linearized with Sal1 or pKSGFP5 as described in (11)]. These reactions were supplemented with 100 µM unlabelled UTP and carried out at 37°C. Where oligonucleotide or '*mir*Vana' templates were used, the only source of UTP was from the radioactively labelled solution ([final] = ~3 µM). Templates to make probes for mmu-mir-292-as, mmu-mir-294, hsa-mir-21, hsa-mir-16 were prepared using the *mir*Vana™ miRNA Probe Construction Kit (Ambion, USA) with oligonucleotides aagtgccgccaggttttgagtgtcctgtctc (mmu-mir-292as), aaagtgc ttccctttgtgtgtcctgtctc (mmu-mir-294), tagcagcacgtaaatatt ggcgcctgtctc (hsa-mir-16) and tagcttatcagactgatgttgacctg tctc (hsa-mir-21) respectively according to the manufacturer's instructions. Resulting templates were used for RNA probe synthesis following manufacturer's instructions. The probes for ath-mir-159b and pi-R1 were prepared by annealing oligonucleotides aagagctcccttcaatccaaacctatagtgagtcgtatta (ath-mir-159) or tgacatgaacacaggtgctcagatagctttcctatagtgagtcgtatta (pi-R1) with the oligonucleotide taatacgactcactatagg. These were used at 250 nM as templates for *in vitro* transcription with $\alpha$-$^{32}$P UTP by T7 RNA polymerase at 22°C.

All *in vitro* transcriptions were treated with TurboDNAse® (Ambion) prior to addition to the hybridization solution to eliminate the DNA template.

End-labelling of oligonucleotides: (1 pmol per labelling) with or without LNA modifications were carried out with T4 polynucleotide kinase and $\gamma^{32}$P ATP (6000 Ci·m mol$^{-1}$; 10 mCi·ml$^{-1}$). Pre-designed LNA oligonucleotides for hsa-mir-21 and hsa-mir-16 were obtained from Exiqon (Denmark). Unmodified oligonucleotide probe sequences were 5′ tcaacatcagtctgataagcta 3′ (hsa-mir-21) and 5′ cgccaatatttacgtgctgcta 3′ (hsa-mir-16) (Sigma).

### Hybridization

Prehybridization and hybridization of both EDC and UV cross-linked membranes were carried out in 2× SSC, 1% SDS and 100 µg·ml$^{-1}$ sheared, denatured salmon sperm DNA. The exact temperature used varied according to the experiment but was between 40 and 60°C. Post-hybridization washes were in 0.2 or 0.1× SSC, 0.2% SDS at temperatures ranging from 40 to 60°C. Membranes were stripped off the probe by placing them in a solution of 10 mM Tris/HCl (pH 8.5 at 25°C), 5 mM EDTA, 0.1% SDS at 100°C for 1 min. Removal of probe was assessed by re-exposure of the membrane for at least as long as any previous exposure. All radioisotopic images were recorded using a FLA-5000 system (Fuji) with Aida Image Analyser software.

## RESULTS

### Extended UV irradiation reveals a discrepancy between retention of small RNA and detection by hybridization

Total RNA extracted from wild-type *A. thaliana* (Columbia) flowers was separated by denaturing PAGE in six lanes with 20 μg RNA per lane. Each adjacent lane was loaded with $\gamma^{32}$P-labelled marker RNA. RNA was transferred from the gel to a sheet of Hybond NX as described in methods and the membrane was cut into six strips, each with a single lane of *A. thaliana* RNA and a marker RNA lane. These strips were exposed to increasing doses of UV irradiation followed by hybridization with a probe complementary to the previously characterized miRNA, ath-mir-159b (16). The signal obtained from each marker lane indicated the degree to which the RNA was robustly linked to the membrane irrespective of conformation or chemical integrity of the RNA. Figure 1 shows that there was a more sensitive dependence on UV dose for RNA of >40 nt than for longer RNA. The optimum dose for RNA <40 nt in length was 0.48 J. In contrast, the optimum UV dose for subsequent detection by hybridization of the miRNA in the adjacent lanes was between 0.12 and 0.24 J. The dose delivered by the manufacturer's 'autocross-link' presetting is 0.12 J and is recommended for optimum detection of mRNA. Increasing the dose beyond 0.24 J progressively reduces the ability to detect the miRNA despite a continued increase in overall RNA retention.

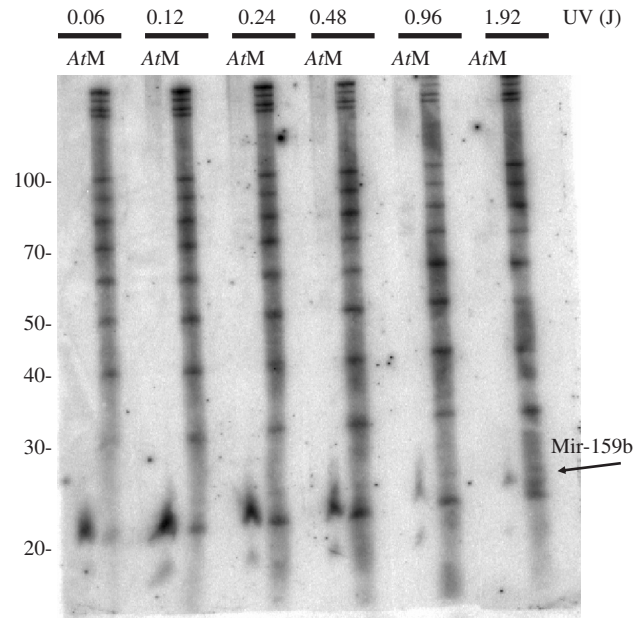


**Figure 1.** The effect of varying UV dose on the detection of small RNA. Here, 20 μg per lane of the same RNA preparation from *A. thaliana* flowers was separated by electrophoresis in six equivalent lanes (*At*). γ-$^{32}$P ATP-labelled Decade® marker RNA was run in each adjacent lane (M). Following electrophoresis, RNA was transferred to Hybond NX® membrane, the membranes then placed in the chamber of a 'Stratalinker' (Stratagene) and irradiated with the indicated doses of UV. The membranes were then placed in the same hybridization bottle and the RNA hybridized at 50°C with a $^{32}$P–UTP-labelled RNA complementary to the ath-mir-159b sequence generated by *in vitro* transcription from an oligonucleotide template. The membrane was washed in 0.1× SSC/0.1% SDS at 50°C and the image detected by phosphorimaging.

### Improved detection of plant siRNA using EDC-mediated cross-linking

We tested whether small RNA from biological samples could be cross-linked to nylon hybridization membranes using the water-soluble carbodiimide EDC and also whether this improved their subsequent detection by hybridization compared to RNA cross-linked by UV. Untreated and dephosphorylated total RNA from a line of transgenic *N. benthamiana* that synthesizes GFP siRNA and from non-transgenic *N. benthamiana* was analysed. Half of each sample was loaded twice on the same gel. The gel was stained after electrophoresis to confirm equal loading (Supplementary Data, Figure 1) and phosphorimaging of both the gel and the membrane was carried out to demonstrate equal and efficient transfer of the $\gamma^{32}$P-labelled marker RNA (Supplementary Data, Figure 2). The membrane was cut into two equal halves and, after UV or EDC cross-linking, the immobilized RNA was hybridized with an α-$^{32}$P-labelled antisense GFP RNA probe. Figure 2a shows that slightly more (2–3-fold) marker RNA (20–40 nt incl.) was retained after cross-linking with EDC compared to UV. The RNA markers used (Decade®; Ambion) were labelled with T4 polynucleotide kinase and $\gamma^{32}$P-ATP and so should react in the EDC-cross-linking step in the same way as siRNA and miRNA that have 5′ terminal phosphates. However, there was an ~30-fold improvement in the detection of GFP siRNA by hybridization. EDC cross-linking of siRNA was dependent on terminal phosphorylation as the signal was almost completely eliminated by treatment of the RNA with alkaline phosphatase prior to electrophoresis.

We also tested whether EDC improved the detection of natural siRNA corresponding to the TS SINE retrotransposon of the plant genus *Nicotiana*. It has been demonstrated previously that siRNA corresponding to this type of retrotransposon can be detected in leaves (8). Quantification of the phosphorimage shown in Figure 2b indicated that compared to UV, EDC enhanced the cross-linking of marker RNA larger than 40 nt by only 1.2-fold but by 2.7-fold for marker RNA of 40 nt or less. EDC cross-linking improved the detection of TS SINE siRNA by over 30-fold. As with GFP siRNA, the cross-linking by EDC (but not UV) was dependent on terminal phosphorylation. TS siRNA were detected using EDC cross-linking from as little as 0.5 μg total RNA (Figure 2b). This signal increased in direct proportion to the amount of RNA loaded.

### Improved detection of mammalian miRNA and temperature dependence with EDC cross-linking

To test whether EDC also improved the detection of mammalian miRNA, we compared the detection, after cross-linking with EDC or UV, of two different miRNA, mmu-mir-292as (mmu-miR-292-3p) and mmu-mir-294, that were originally shown to accumulate in murine ES cells (17). Because miRNA generally accumulate as a

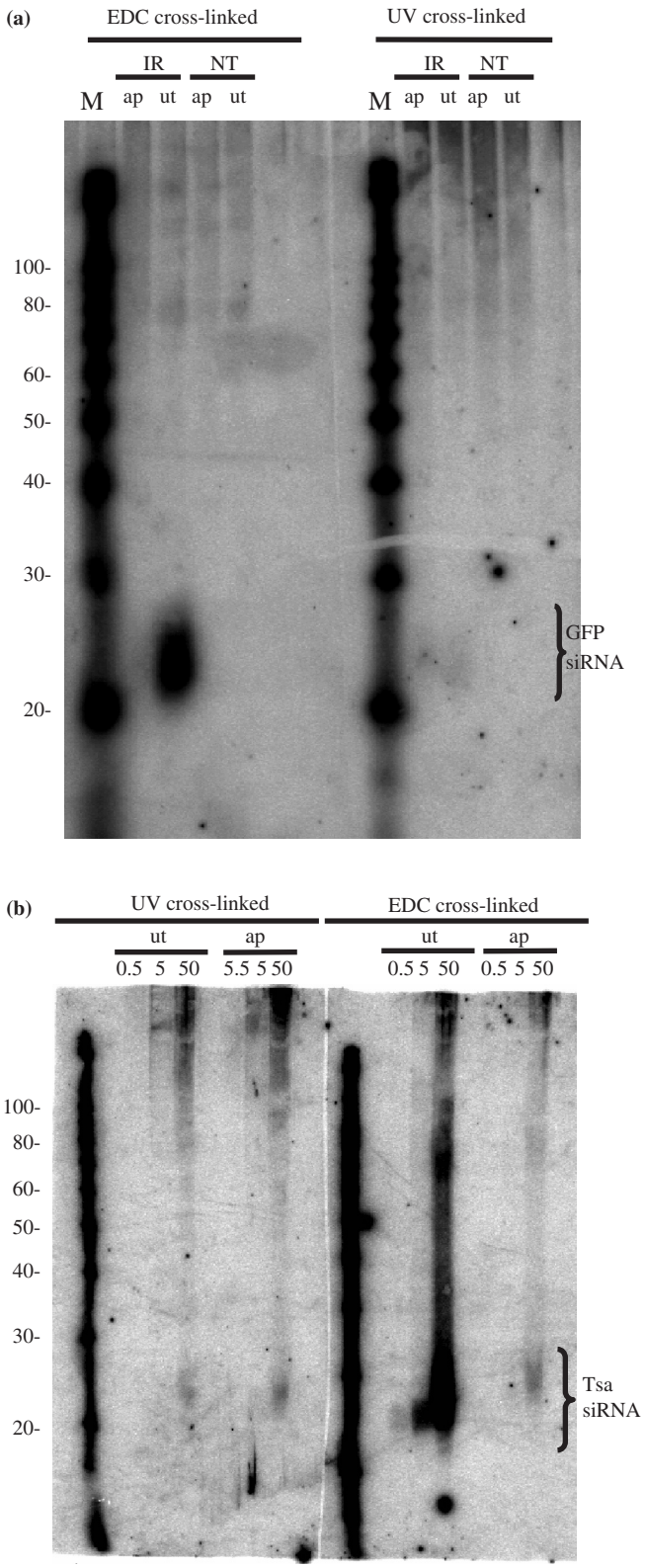


**Figure 2.** EDC cross-linking improves detection of plant siRNA. (**a**) RNA was extracted from leaves of transgenic *N. benthamiana* line GFP 16C/GFPi that transcribes a partial GFP inverted repeat (IR) or from non-transformed *N. benthamiana* (NT). From each sample, 5 μg of untreated RNA (ut) or 5 μg of RNA treated with alkaline phosphatase (ap) were run in adjacent lanes. The gel was stained with SybrGold® to record the equal loading, and then blotted onto Hybond NX® membranes prior to cross-linking. RNA was cross-linked with either EDC at 60°C for 2 h or with 0.24 J UV. Both filters were hybridized in the same solution with a complementary RNA probe specific for GFP. The membrane was washed in 0.2× SSC/0.2% SDS at 50°C and the image detected by phosphorimaging. (**b**) RNA was extracted from leaves of non-transformed *N. benthamiana*. Half of this RNA was treated with alkaline phosphatase and 0.5, 5 and 50 μg of both untreated (ut) and phophatased (ap) RNA run on the same gel. The gel was stained with EtBr to record loading and then the RNA was blotted onto a Hybond NX® membrane. RNA was cross-linked with either EDC at 60°C for 1 h or with 0.24 J UV. Both membranes were hybridized in the same bottle with a full-length TS SINE probe. The membranes were washed in 0.1× SSC/0.1% SDS at 50°C and the image detected by phosphorimaging.

single molecular species, this also allowed us to examine more accurately the effect of varying the time and temperature of EDC cross-linking efficiency. Low molecular weight RNA from differentiated murine ES cells was separated by electrophoresis, the gel stained with EtBr (Supplementary Data, Figure 3) and blotted onto Hybond NX® membrane. In this experiment, the UV treatment cross-linked the $\gamma^{32}$P-labelled RNA markers as efficiently as the optimum EDC treatment (Figure 3). However, as for the plant siRNA, there was substantially enhanced detection of both mmu-mir-292as (50 fold) (Figure 3a) and mmu-mir-294 (Figure 3b) using EDC cross-linking compared to UV. Mmu-mir-294 was not convincingly detected using UV cross-linking, so the manifest improvement in detection was not quantified. The optimum conditions appeared to differ slightly for each miRNA but are between 50 and 60°C for 1–2 h. Surprisingly, the EDC cross-linking of the $\gamma^{32}$P-labelled marker RNA varied less with time and temperature compared to the miRNA.

### Improved detection of piRNA using EDC-mediated cross-linking

A new class of small RNA related to microRNA and siRNA, which accumulate in rodent testes, was recently described by several groups [reviewed in ref. (3)]. These are known as piRNA and are in the range of 29–31 nt in length. Figure 4 shows that EDC cross-linking improves the detection of one of these, pi-R1 (18) compared to UV cross-linking. The increase in sensitivity was 20–25-fold, slightly less than that observed for siRNA and microRNA. We observed similar improved detection using EDC compared to UV of the two other piRNA that we tested (data not shown).

### Comparison of LNA, DNA and RNA probes with EDC versus UV cross-linking

Three methods that are commonly used to prepare hybridization probes for small RNA detection were compared for their compatibility with EDC versus UV cross-linked RNA.

(i) Pre-designed, oligodeoxynucleotides incorporating 'locked nucleic acid' (LNA®) modifications. The sequences were entirely complementary to the target miRNA and were end-labelled with γ-$^{32}$P ATP by T4 polynucleotide kinase. LNA-containing oligonucleotides have been shown to offer much greater

sensitivity for the detection of miRNA by standard small RNA northern blotting using UV for cross-linking (19).

(ii) Unmodified oligodeoxynucleotides complementary to the target miRNA and that were end-labelled with $\gamma$-$^{32}$P ATP by T4 polynucleotide kinase.

(iii) RNA oligonucleotides transcribed *in vitro* with $\alpha$-$^{32}$P UTP from templates created with the mirVana® kit (Ambion). The RNA probes generated are fully complementary to the target miRNA but additionally have the 5′ leader sequence GGGAGACAGG.

Here, 5 µg of the same sample of HeLa cell total RNA was run in six lanes of the same denaturing gel. $^{32}\gamma$-P-end-labelled Decade® marker RNA was run in each adjacent lane. The gel was stained with EtBr to confirm equal loading (Supplementary Data, Figure 5). After blotting, membranes were cross-linked with either EDC or UV. Probes were prepared by the above three methods for the detection of two human miRNA: hsa-mir-21 (Figure 5a) and hsa-mir-16 (Figure 5b).

The blots were first hybridized with the three differently synthesized hsa-mir-21 probes. After exposure, the blots were stripped, exposed to ensure complete removal of probe (Supplementary Data, Figure 6) and re-hybridized with the three differently synthesized probes for hsa-mir-16. No signal was detected with the end-labelled unmodified oligonucleotides probably because of the relatively low amount of total RNA used (5 µg). LNA probes with EDC-cross-linked RNA produced the strongest signal for both miRNA tested although, for hsa-mir-16, this was only slightly greater than if using a RNA probe for EDC-cross-linked RNA.

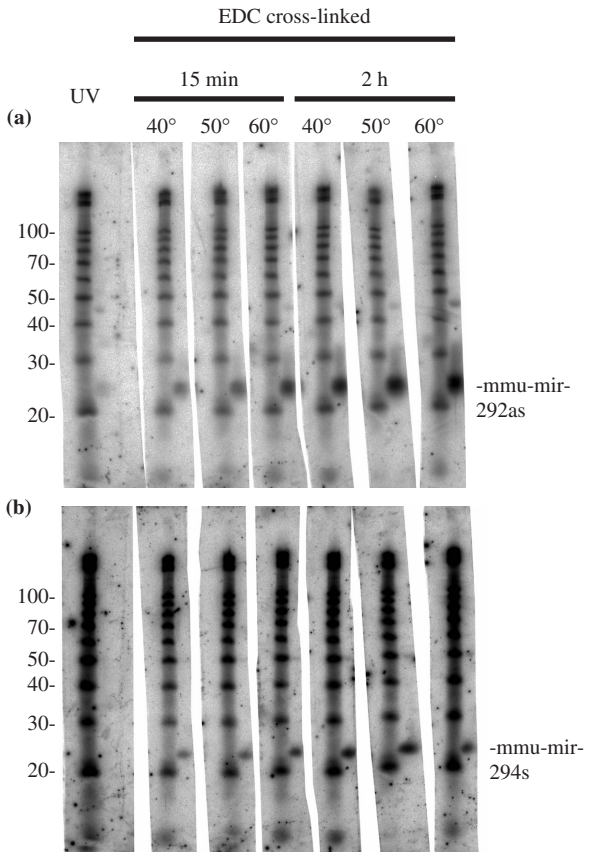


**Figure 3.** EDC cross-linking improves detection of mammalian miRNA. Identical 5-µg aliquots of low molecular weight RNA from differentiating murine embryonic stem cells were loaded in multiple lanes of the same denaturing polyacrylamide gel. The same volume of $^{32}$P-labelled RNA markers was loaded in each adjacent lane. After electrophoresis and transfer to one sheet of nylon membrane, strips containing one individual sample RNA lane and one adjacent marker RNA lane were cut and cross-linked with 0.24 J UV or EDC at 40, 50 and 60°C for 15 min or 2 h. All membranes were then hybridized in the same bottle with a probe to detect (**a**) mmu-mir-292as. After exposure, the membranes were stripped and re-probed for mmu-mir-294 (**b**). The membrane was washed in 0.2× SSC/0.1% SDS at 50°C and the image detected by phosphorimaging.

## DISCUSSION

Recent discoveries of thousands of novel, small RNA and increasing appreciation of their diverse biological functions (1–3,9,20) have led to a demand for improved experimental procedures to study these molecules. Northern blot analysis remains one of the most convincing ways to determine the presence, size and abundance of a small RNA but it is generally regarded as one of the least

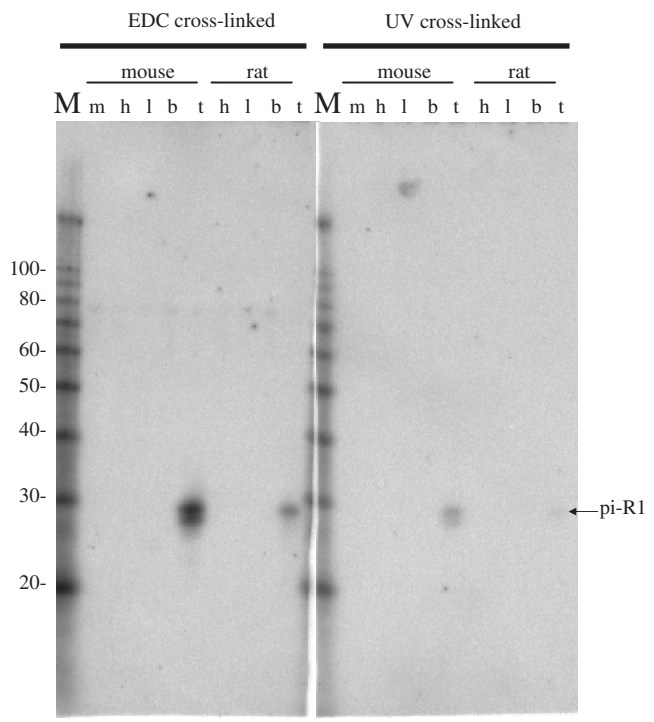


**Figure 4.** Improved detection of piRNA using EDC-mediated cross-linking. Total RNA was extracted from mammary gland (m), heart (h), liver (l), brain (b) and testes (t) of adult mice and rats. Here, 10 µg of each sample was run in duplicate on a 10% polyacrylamide gel overnight at 60 V. RNA in the gel was stained by EtBr (Supplementary Data, Figure 4) and then transferred by electroblotting to Hybond NX®. The membrane was cut into two equal halves, each containing the same set of sample RNA. One-half was cross-linked using EDC for 2 h at 60°C. The other half was cross-linked with UV (0.12J). The membranes were hybridized in same bottle overnight with an RNA probe to detect mouse pi-R1 at 50°C in 2× SCC/1% SDS, washed at 50°C in 0.1× SSC/1% SDS and exposed for 24 h.

sensitive analytical methods (9). We hypothesized that the UV cross-linking step in this method, which was originally developed for the efficient detection of mRNA, might not be optimum for RNA of only 20–30 nt in length. Therefore, we tested whether varying the dose of UV delivered by a standard UV cross-linking cabinet could improve the detection of a typical small RNA (a 21-nt miRNA). Figure 1 shows that the optimum UV dose for the detection of that miRNA was, in fact, very similar to the recommended dose for mRNA (~0.12 J) and that increasing the UV dose resulted in progressively less sensitive miRNA detection. Despite the reduction in detection of miRNA by hybridization beyond 0.12 J, direct detection of the 20 and 30 nt radioactive RNA markers increased, reaching an optimum at a 4-fold higher UV dose. Thus, the optimum UV dose for the detection of small RNA by hybridization was not the same as the dose that maximally cross-linked the RNA. Several possible events might account for this including UV-degradation of the functional groups required for base-pairing, fragmentation of the RNA and excessive cross-linking limiting access for complementary probes. We tested three other UV-cross-linking cabinets (all from Stratagene) located within other departments in our institute and found there was no difference in their performance compared to the unit used in this study with respect to the detection of a miRNA or in the cross-linking of marker RNA (Supplementary Data, Figure 7). We concluded that when using UV as a cross-linker, there was a trade-off between increasing retention of small RNA on a membrane and progressively reducing the ability of that immobilized RNA to form stable double-stranded hybrids. Therefore we investigated alternative methods for cross-linking RNA to hybridization membranes.

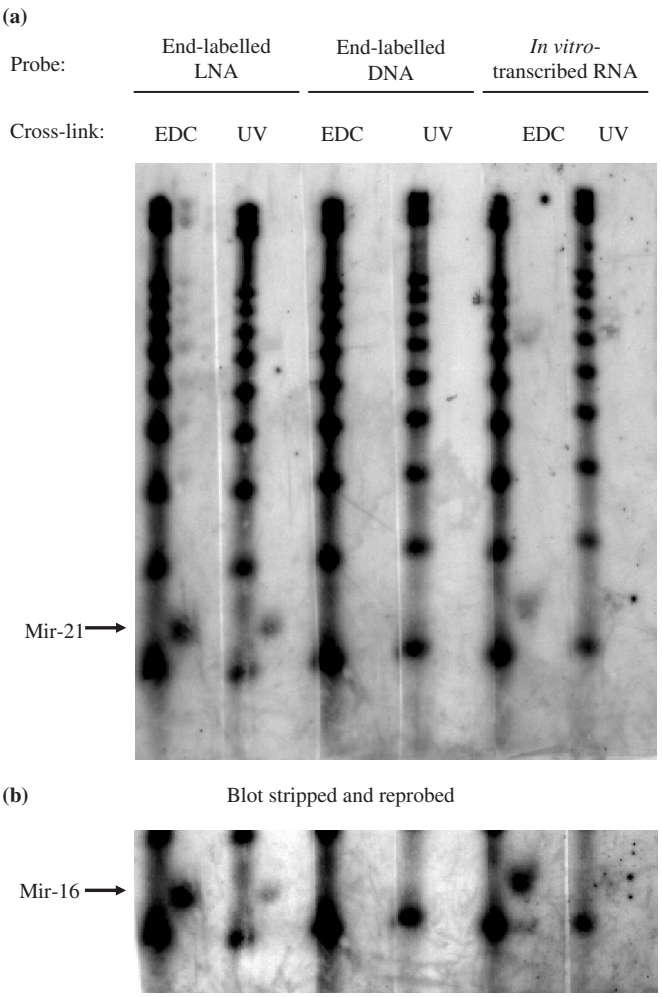


**Figure 5.** A combination of LNA probe and EDC cross-linking provides the most sensitive detection of miRNA. Identical 5-μg aliquots of total RNA from HeLa cells were loaded in multiple lanes of the same denaturing polyacrylamide gel. The same volume of $^{32}$P-labelled RNA markers was loaded in each adjacent lane. After electrophoresis and transfer to one sheet of Hybond NX® nylon membrane, six strips each containing one individual sample RNA lane and one adjacent marker RNA lane were cut. Three were cross-linked with 0.24 J UV and three with EDC at 60°C for 2 h. Pairs of UV and EDC cross-linked membranes were hybridized with probes designed to detect (**a**) hsa-mir-21 and, after stripping, (**b**) hsa-mir-16. Probes were LNA or DNA oligonucleotides end-labelled with γ-$^{32}$P ATP or RNA transcribed *in vitro* with α-$^{32}$P UTP. Hybridizations were carried out at 40°C and blots washed in 0.2× SSC/0.2% SDS at 50°C and the image detected by phosphorimaging.

Ideally, for detection by hybridization, small RNA would be efficiently coupled to a support in such a way that the entire polynucleotide was freely available for base-pairing with the complementary probe. In principle, functional groups not located on the heterocyclic bases and that are specific to the 5′-end or 3′-end would be the most suitable reactants. In animals, small regulatory RNA such as siRNA and miRNA generally have 5′ terminal monophosphates and a 2′, 3′ *cis*-diol at their 3′-terminus (21). However, some 3′ modifications of small RNA have been described in insects and plants (7,22), therefore cross-linking through the 5′-terminal phosphate would seem to offer the greatest flexibility.

EDC has been used to cross-link 5′-phosphorylated oligodeoxyribonucleotides to primary amines on controlled pore glass supports (23) and this resulted in superior hybridization properties compared to nitrocellulose-bound DNA (24). EDC has also been used to couple phosphorylated oligodeoxyribonucleotides that were directly pumped onto the surface of the negatively charged nylon membrane, Biodyne C® (25). However, it was not obvious that a similar process could be used for cross-linking small RNA from biological samples because the coupling efficiency was not indicated: efficient coupling would be critical for a successful northern blot adaptation. We tested whether EDC could be used as an efficient cross-linker of several different small RNA from biological samples to standard, neutral nylon hybridization membranes (Hybond NX®) after deposition by electro-blotting from denaturing polyacrylamide gels. Figure 2 shows that detection of plant siRNA by hybridization was enhanced by ~40-fold after EDC cross-linking compared to UV. Small (20–40 nt) radioactive, marker RNA was cross-linked only 2–3-fold more efficiently by EDC than UV in the same experiment. The proportionally greater enhancement of detection by hybridization may have resulted from reduced degradation and/or improved presentation of EDC-cross-linked RNA. This result also indicated that contaminating molecules likely to be present

in RNA preparations from biological sources did not significantly interfere with cross-linking. The observed dependence on terminal phosphorylation is consistent with the expected formation of a phosphoramidate bond between the RNA and the nylon. For the experiment shown in Figure 2, this was likely to be 5′ phosphorylation according to the known properties of siRNA. We have not tested whether the detection of RNA with 3′-terminal phosphates would similarly benefit from EDC cross-linking. Staining of gels with EtBr prior to transfer did not reduce the enhancement of hybridization signal obtained with EDC cross-linking (data not shown). siRNA detection increased in proportion to the amount of RNA loaded up to the maximum tested (50 μg of total RNA, Figure 2b). This suggests that cross-linking sites on the membrane were not becoming saturated and that the membrane might possibly accommodate larger amounts of RNA without a reduction in cross-linking efficiency. Thus, very rare small RNA might be detected if sufficient quantities of RNA enriched for low molecular weight species were available.

Figure 3 shows that EDC cross-linking also greatly enhances the detection of miRNA from mammalian cells. This experiment also tested the effect of varying time and temperature on the EDC-cross-linking step. Similar optimum conditions (2 h at 50 or 60°C) were identified for hsa-mir-292as and hsa-mir-294. The same optimum conditions were found for two other miRNA tested (data not shown). In this experiment, EDC cross-linking did not significantly improve the retention of marker RNA compared to UV indicating again that increased miRNA detection sensitivity is largely the result of improved RNA quality and/or positioning on the membrane. The detection of piRNA was also improved using EDC cross-linking compared to UV although by only 20–25-fold (Figure 4) using these conditions.

Probes that incorporate LNA modifications have been shown to substantially enhance the detection of miRNA in standard northern blot protocols (i.e. with UV cross-linking) (19). Figure 5 shows that the most sensitive detection of two different mammalian miRNA was achieved with a combination of LNA probe and EDC cross-linking. However, for one of those microRNA, the use of EDC cross-linking with an inexpensive, *in vitro* transcribed RNA probe was nearly as effective.

In principle, neutral nylon membranes from other manufacturers might be as suitable as Hybond NX® but we have not extensively tested this. One positively charged membrane tested (Hybond N+®) gave substantially poorer results than the neutral membrane (data not shown). Although we did not establish an upper limit on the amount of small RNA that can be cross-linked using EDC to existing neutral nylon membranes (per $mm^2$), saturation will inevitably occur at some point. Therefore, future improvements to the described process could possibly be made by fabrication of membranes with increased surface amine content. However, care would have to be taken not to reduce the initial, very efficient non-covalent adsorption of RNA at the point of blotting/transfer that neutral nylon membranes provide (data not shown).

We have briefly tested other standard hybridization conditions such as those with buffers containing high concentrations of SDS or formamide and found them to be equally compatible with EDC-cross-linked membranes (data not shown).

In summary, we have described a simple, inexpensive alternative to UV cross-linking that greatly improves the detection of small, terminally phosphorylated RNA such as siRNA, miRNA and piRNA by northern blotting. It is likely that the detection of other related small RNA will also benefit from this procedure.

## SUPPLEMENTARY DATA

Supplementary data is available at NAR online.

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