

## TECH TIP #210

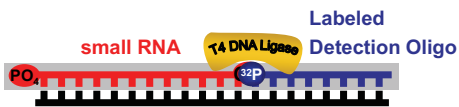
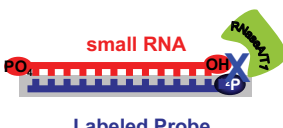
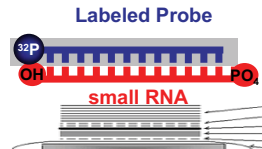
### miRtect-IT™: A Novel Method for Small RNA Detection

#### Introduction

The recent discovery and characterization of small non-protein-coding regulatory RNAs, such as microRNAs (miRNAs), PIWI-associated RNAs (piRNAs and rasiRNAs), short interfering RNAs (siRNAs), *trans*-acting siRNAs (*ta*-siRNAs), and other families of short RNAs has led to a rapid expansion of research directed at elucidating their expression patterns and regulatory functions<sup>(1,2)</sup>. Currently, Northern blotting is the standard method for the detection of small RNAs, because it allows direct comparison of the quantity of small RNA between different samples. However, major drawbacks of Northern blotting are time consuming procedures and poor sensitivity, especially when monitoring expression of short nucleotide sequences. Despite the improvements in detection sensitivity provided by Locked Nucleic Acid (LNA) substituted probes<sup>(3)</sup>, Northern blotting requires relatively large amounts of starting material and involves multiple handling steps (Table 1).

Another approach for small RNA detection is based on the ribonuclease protection assay (RPA), which takes advantage of liquid hybridization kinetics to improve detection sensitivity (Table 1). Both Northern blotting and RPA determine the amount of small RNA by measuring the amount of the labeled probes that are non-covalently hybridized to the small RNA, providing a relative quantification of small RNA levels. In addition, the critical experimental points that determine the assay efficiency, which are the hybridization and wash steps in the Northern Blot assay and the hybridization and ribonuclease treatment in RPA, usually require optimization because insufficient treatment may lead to non-specific detection while excess treatment may compromise the detection sensitivity.

Table 1. Comparison of amplification-independent assays for small RNA detection.

	miRtect-IT™ Labeling and Detection	Ribonuclease Protection Assay	Northern Blot
			
assay principle	direct labeling and detection of ligated small RNAs	indirect detection of the labeled probes protected by small RNAs from ribonuclease digestion	indirect detection of the labeled probes hybridized to small RNAs
Hybridization	in-solution hybridization	in-solution hybridization	membrane hybridization
probe labeling	end labeled with radioactivity	end labeled with radioactivity	end labeled with radioactivity
starting materials	nano- to microgram quantities of total RNA	nano- to microgram quantities of total RNA	microgram quantities of total RNA
assay protocol	add-and-incubate	ribonuclease treatment and RNA precipitation	membrane transfer, hybridization and wash
sample separation	denaturing PAGE*	denaturing PAGE*	denaturing PAGE*
detection	X-ray film or phosphorimager	X-ray film or phosphorimager	X-ray film or phosphorimager
time from start to finish	6 hr-1 day	1-2 days	2-3 days

\* PAGE: polyacrylamide gel electrophoresis

Here, USB offers a novel approach for direct labeling and detection of small RNAs. The miRtect-IT™ technique takes advantage of liquid hybridization kinetics to improve detection sensitivity, but without a procedure that requires optimization. Similar to Northern blotting, miRtect-IT™ does not require specialized equipment or any amplification step, and thus allows direct and accurate measurement of specific small RNAs.

### miRtect-IT™ Small RNA Detection Kit

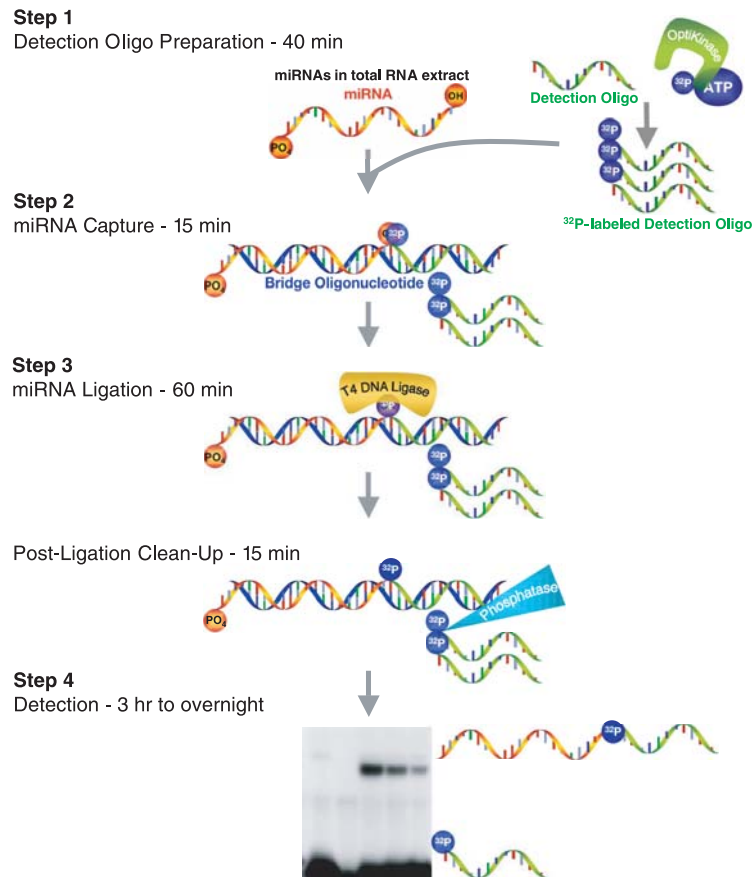
The miRtect-IT™ Kit uses a unique strategy for direct labeling, detection and quantification of small RNAs from total RNA using splinted ligation<sup>(4)</sup>. The splinted-ligation technology is a nucleic acid hybridization assay that uses a small RNA-specific Bridge Oligonucleotide (Oligo) with perfect Watson-Crick complementarity to the small RNA and a 5'-end radiolabeled Detection Oligo (Fig. 1). The captured small RNA and the Detection Oligo are then covalently linked with T4 DNA Ligase to generate a one-to-one ratio of labeled molecule per small RNA. In addition, while the labeled phosphate provided by the Detection Oligo is internal and therefore insensitive to the phosphatase clean-up treatment, the label present on the unligated Detection Oligo can be removed by incubation with the Clean-Up mix after the ligation step. Following the splinted-ligation reaction, labeled small RNAs carrying a 14 nucleotide extension and any residual labeled Detection

Oligo can then be separated by denaturing gel electrophoresis and visualized by autoradiography.

The miRtect-IT™ assay is appropriate to detect all known small RNA sequences, provided they have a 3' hydroxyl group to create a covalent phosphodiester linkage between the small RNA and the 5' phosphate on the Detection Oligo. The miRtect-IT™ assay is suitable for the labeling of small RNAs with modified nucleic acids at 5' ends. Although 3' end modifications may interfere with the ligation reaction, small RNAs with their 3' ends modified by 2'-O-methylation (*e.g.* plant miRNAs and germ-line piRNAs) can be reliably detected by the miRtect-IT™ Kit (Fig. 2).

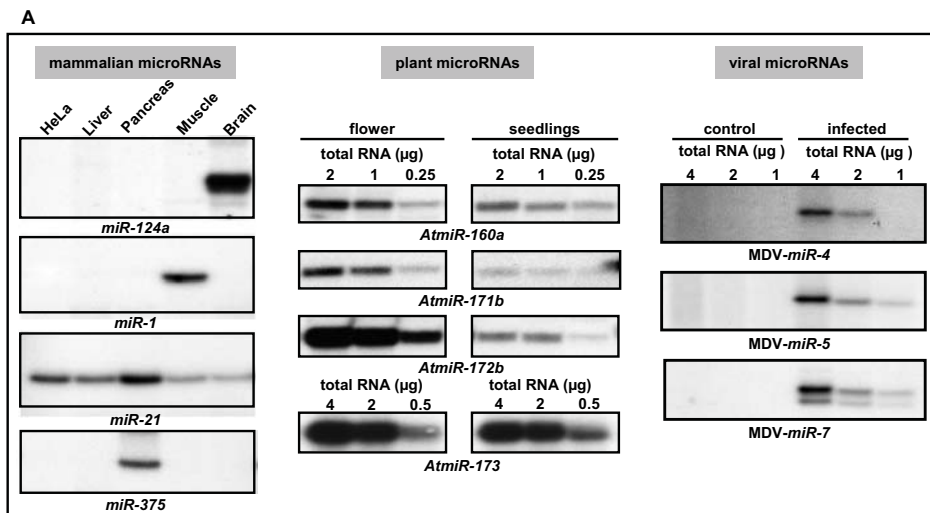
### miRtect-IT™ Kit Detection Sensitivity

To examine the miRtect-IT™ Kit detection sensitivity, we compare the miRtect-IT™ assay to Northern blotting for miRNA detection and show that the miRtect-IT™ assay is approximately 50 times more sensitive than Northern blotting (Fig. 3A). The miRtect-IT™ assay has a linear detection range of 0.2 to 20 femtomoles (Fig. 3B). This assay is also quantitative. In order to determine the amount of a small RNA in a sample, a dilution series of a synthetic oligoribonucleotide of known concentration is analyzed in parallel with the sample. A linear standard curve can be generated from which the concentration of small RNA in the sample can be determined.



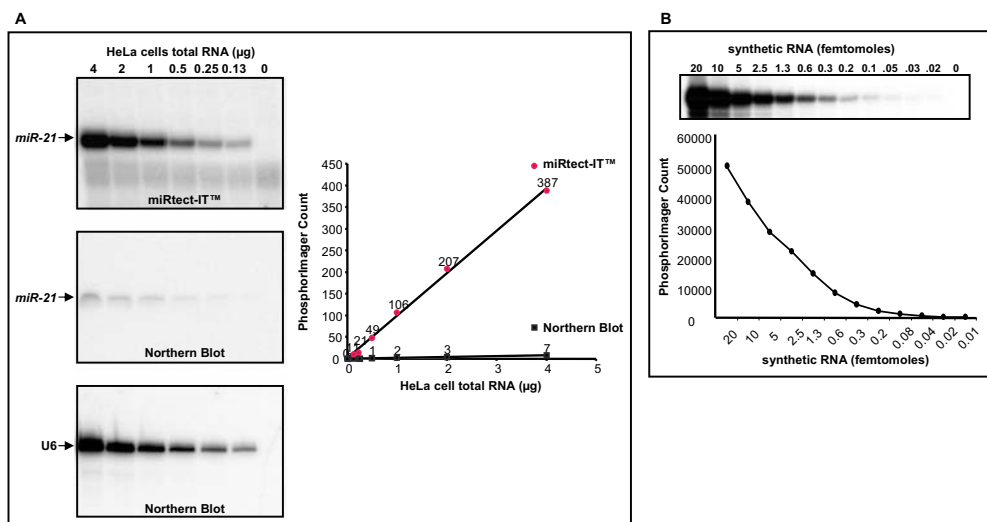
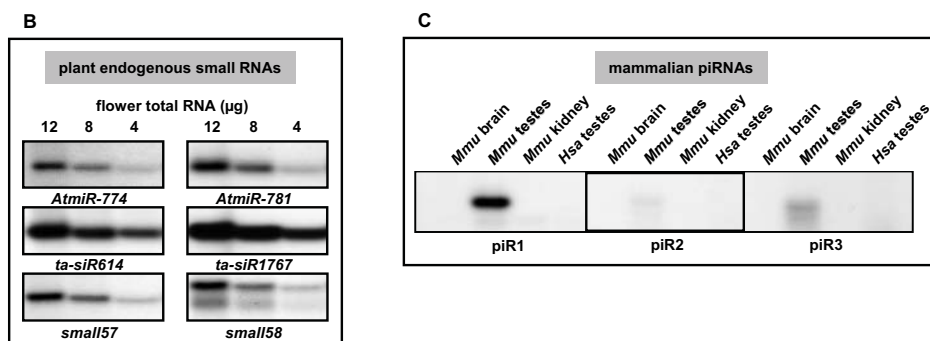
**Fig. 1 Flowchart of miRtect-IT™ small RNA Labeling and Detection Kit procedure.**

The protocol is divided into four steps: **Step 1** Labeling of the Detection Oligo; **Step 2** Capturing of the Detection Oligo and the small RNA with a Bridge Oligo through base-pairing; **Step 3** Joining of the Detection Oligo to the small RNA by ligase followed by a clean-up reaction of un-ligated Detection Oligo; **Step 4** Fractionation on a denaturing gel.



**Fig. 2 miRtect-IT™ Kit results on expression analyses of various classes of endogenous regulatory small RNAs.**

Human, plant and viral miRNAs (A), low abundant Arabidopsis miRNAs, *ta*-siRNAs and endogenous small RNAs (B), mouse piRNAs (C) were detected using the indicated amounts of total RNA in A and B. Total RNA were used at 250 ng and 4  $\mu$ g for A-mammalian miRNAs and in C, respectively. The image was developed after a 2–48 hr exposure. Reprinted with permission.



**Fig. 3 Sensitivity and quantitative measurement of small RNAs by the miRtect-IT™ Kit.**

Comparison of the detection sensitivity of miRtect-IT™ and Northern blotting using the indicated amounts of HeLa cell total RNA for the presence of *hsa-miR-21* (A). The Northern blot was also probed for U6 snRNA detection as a loading control. Detection sensitivity of the miRtect-IT™ assay using serial two-fold dilutions as indicated of a synthetic 21 nucleotide *hsa-miR-21* RNA oligonucleotide (B). The signals were quantified with a Storm PhosphorImager (GE Healthcare). Reprinted with permission.

### miRtect-IT™ Kit Applications

The miRtect-IT™ Kit has been used to detect different classes of small RNAs from unfractionated RNA samples<sup>(4)</sup> and to determine the miRNA distribution on polyribosomes after hypertonic stress<sup>(5)</sup>. The miRtect-IT™ Kit has also been validated for expression profiling of the following biological small RNA targets: mammalian, plant and viral miRNAs, piRNAs, plant-specific small RNAs, *ta*-siRNAs, ribosomal

RNA, small nuclear (sn)RNAs and small nucleolar (sno)RNAs. The results obtained using the miRtect-IT™ technology have been in agreement with published studies that have characterized organ-specific miRNA expression in animals, regulation of plant miRNAs in flower and seedlings, and testis-specific expression of piRNAs (Fig. 2).

## Conclusions

The miRtect-IT™ Kit is a novel technique for the labeling and detection of small RNAs by splinted–ligation technology. The miRtect-IT™ assay is unique because it can directly label small RNAs from a non–enriched total RNA sample without an amplification reaction, making it ideal for accurate detection and quantification of subfemtomole amounts of small RNAs. The miRtect–IT™ assay does not require specialized equipment, and is significantly faster and more sensitive than Northern blotting. The assay set-up is simple and thus allows easy processing of multiple samples. The kit has been successfully validated to study expression profiling of various classes of biological small RNAs. For complete details, and to download the protocol, please visit [www.usbweb.com/mirtect-it](http://www.usbweb.com/mirtect-it).

## References

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## Related Products

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PrepEase® Plant RNA Spin Kit	78770/78771

### Buffers for RNA Applications

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Water, RNase-Free	71783
Water, RNase-Free, DEPC Treated	70783

### Gel Electrophoresis

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RNA Ladder, 0.1 – 1 kb	76722
RNA Markers, 0.2 – 10 kb	76724
RapidGel 40% Liquid Acrylamide Stock Solution	75848
Urea	75826
Glycerol Tolerant Gel (GTG) Buffer, 20X Solution	75827
TBE Buffer, 5X Solution	75891
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