

# miRtect-IT™ miRNA Labeling and Detection Kit

**Product Number 76400**  
**100 Assays**

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†miRtect-IT™ miRNA Labeling and Detection Kit – Patent pending.

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## **STORAGE**

Store at -20°C.

**Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.**



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## QUALITY CONTROL

The miRtect-IT™ miRNA Labeling and Detection Kit is functionally tested for miRNA detection using total RNA and the Positive Control following the protocol described in this manual.

Each kit component is free of endonuclease, exonuclease, or ribonuclease contamination. Properly handled and stored components are guaranteed for optimal performance for at least 6 months from the date received.

## SAFETY WARNINGS AND PRECAUTIONS

**Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.**

Caution: All chemicals should be considered as potentially hazardous. We, therefore, recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as lab coat, safety glasses, and gloves. Care should be taken to avoid contact with skin and eyes. In the case of contact with skin or eyes, wash immediately with water. See MSDS (Material Safety Data Sheet) for specific advice.

## INTRODUCTION

### Background Information

MicroRNAs (miRNAs) are a family of 20-23 nucleotide non-coding, small RNAs that regulate gene expression at the post-transcriptional level. Interaction between the miRNA and its mRNA target often results in inhibition of protein synthesis. To date, more than 1,000 miRNAs have been identified in animals and plants according to the miRNA registry. Growing evidence suggests that miRNAs are important regulators of cell division and differentiation as well as human cancer genes<sup>(1,2,3)</sup>. Recently, the discovery of regulatory effects on gene expression has led to numerous studies on the characterization of miRNA function in molecular processes and as possible tools in drug discovery.

Interest in miRNAs and a growing number of small regulatory RNAs has created a demand for novel tools to study expression<sup>(4)</sup>. Presently, Northern blotting is the standard technique for small RNA expression analysis. However, a major drawback of Northern blots is poor sensitivity, especially when monitoring expression of short nucleotide sequences such as miRNAs. In addition, a large amount of total RNA is often required for Northern blots. Despite improvements in detection such as using Locked Nucleic Acid (LNA) probes<sup>(5)</sup>, the procedures for Northern blot assay remain labor intensive and time-consuming.

## Product Description

The miRtect-IT™ miRNA Labeling and Detection Kit is a ligation-based assay that provides a unique approach for detection and quantification of small RNAs<sup>†</sup> such as mature miRNA from total RNA by splinted-ligation technology<sup>(4,6,7)</sup>. The splinted-ligation technology is a nucleic acid hybridization assay that uses a miRNA-specific Bridge Oligonucleotide to form base pairs with the miRNA and a Detection Oligo. The captured miRNA is subsequently ligated to the Detection Oligo with T4 DNA Ligase (Figure 1).

By joining the miRNA to the radiolabeled Detection Oligo, the assay directly labels miRNAs, and avoids the inherent difficulty of detecting small RNAs of 20-32 nucleotides by Northern blots. Quantitative measurement of miRNAs in total RNA samples can be completed within 6 hours.

The kit includes optimized reagents and a detailed protocol for preparing the radiolabeled Detection Oligo and for joining miRNAs with T4 DNA Ligase.

The Bridge Oligonucleotide specific for a miRNA of interest must be obtained separately from a custom oligonucleotide synthesis supplier. (See additional information in Supplementary Information on Bridge Oligonucleotide Design and Preparation on page 12).

## Assay Procedure Overview

miRtect-IT™ miRNA Labeling and Detection Kit is a four-step procedure for direct labeling of miRNA from nanogram to microgram quantities of total RNA (see Figure 1).

### Step 1 Detection Oligo Preparation (40 min)

- 5' End-label Detection Oligo with [ $\gamma$ -<sup>32</sup>P]-ATP and OptiKinase™
- Remove unincorporated isotope with Clean-Up Column

### Step 2 miRNA Capture (15 min)

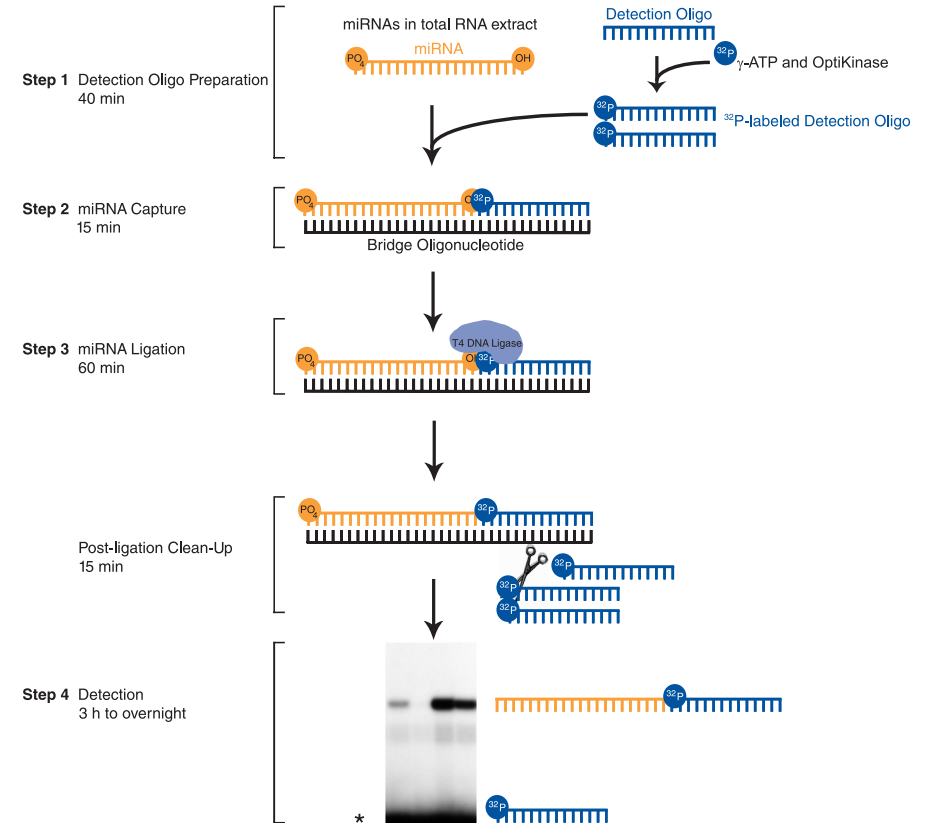
- Capture miRNA and the radiolabeled Detection Oligo with Bridge Oligonucleotide

### Step 3 miRNA Ligation (75 min)

- Ligate the captured miRNA and the radiolabeled Detection Oligo with Ligate-IT™ Premix
- Remove non-ligated Detection Oligo with Clean-Up Mix

### Step 4 Detection (3 hr to overnight)

- Separate ligated miRNA on a 12-15% UREA-polyacrylamide gel
- Visualize by X-ray film or a phosphorimaging system



**Figure 1. miRtect-IT™ miRNA Labeling and Detection Kit assay procedure.**

\* Residual radiolabeled 14 nucleotide Detection Oligo will be present due to incomplete removal of the 5' end-<sup>32</sup>P labeled Detection Oligo.

## COMPONENTS

The miRtect-IT™ miRNA Labeling and Detection Kit should be stored at -20°C in a non-frost-free freezer upon arrival. The kit provides reagents for preparing 5 radiolabeled reactions sufficient for performing 100 detection assays. The kit also includes a Positive Control for 10 assays.

Components	Quantity	Storage Temperature	Component Cap Color
Detection Oligo	10 µl	-20°C	Blue
OptiKinase™	10 µl	-20°C	Blue
10X OptiKinase™ Reaction Buffer	10 µl	-20°C	Blue
Clean-Up Column	5 columns	-20°C	N/A
10X Capture Buffer	3 X 1 ml	-20°C	Red
3X Ligate-IT™ Premix	500 µl	-20°C	Red
Positive Control	10 µl	-20°C	Red
Clean-Up Mix	100 µl	-20°C	Red
Gel Loading Dye	2 X 1 ml	-20°C	White
RNase-Free Water	5 ml	-20°C	White

The Detection Oligo is a specific 14-base oligonucleotide.

The Positive Control is a Premix of 2 oligonucleotides:

1. 22-base synthetic oligonucleotide corresponding to the sequence of a known miRNA
2. 36-base Bridge Oligonucleotide for capturing the 22-base synthetic miRNA (see 1 above).

Together they generate a 36-base labeled product.

## Materials Not Supplied

- Bridge Oligonucleotide (10 pmol, 0.1 pmol/µl in 10X Capture Buffer) [obtained from a custom oligonucleotide synthesis supplier, see Supplementary Information on Bridge Oligonucleotide Design and Preparation on page 12]
- [ $\gamma$ -<sup>32</sup>P]-ATP (6000 Ci/mmol, **150mCi/ml**) [Perkin Elmer, PN NEG035C010MC or GE Healthcare, PN PB15068]
- Low Molecular Weight Marker, 10-100 nt (for size reference of 30-50 nt single-stranded fragments) [USB PN 76410]
- 2.0, 1.5, 0.2 ml microcentrifuge tubes, RNase-Free
- Adjustable precision pipettes
- Disposable aerosol barrier pipette tips, RNase-Free
- Vortex mixer

- Microcentrifuge
- Thermal cycler or waterbath
- 12-15% UREA-polyacrylamide gel [See Supplementary Information page 14]
- Vertical gel electrophoresis system
- X-ray film or phosphorimaging system

## PROTOCOL CONSIDERATIONS

### General Guidelines

- Thaw reagents on ice, mix thoroughly before use and immediately return unused materials to -20°C.
- When preparing working reagents, measure components accurately, mix thoroughly, spin briefly and keep on ice.
- Assemble reactions on ice or at indicated temperature throughout the procedure.
- Follow the general guidelines in the Supplementary Information section to prevent RNase contamination.

### Starting Materials

The amount of total RNA required per assay depends on the abundance of the miRNA of interest. The miRtect-IT™ miRNA Labeling and Detection Kit has a linear detection range of 0.2-20 fmol based on measurement of synthetic miRNAs. The recommended protocol allows up to 8 µl of total RNA or RNA enriched for small RNAs per assay reaction. A typical assay reaction uses 0.5-4 µg of RNA diluted in TE Buffer or RNase-Free Water. See Supplementary Information on page 14, General Considerations for Total RNA Preparation.

## Recommended Assay Setup

### Assay Controls

- Prepare a “**Positive Control**” to assess assay components and procedure by substituting the RNA sample with the supplied Positive Control of miRNA-Bridge Oligonucleotide Premix.
- Prepare a “**No RNA Negative Control**” to assess sample background signal by substituting the RNA sample with RNase-Free Water.
- Suggestion for “**Internal/Loading Control**”
  - Detect miRNAs known to be constitutively expressed in the tested samples.
  - Stain gels for tRNA detection with ethidium bromide or other single-stranded nucleic acid staining dyes.

## PROTOCOL

### A. Detection Oligo Preparation and Clean-Up

The first step is to 5' end-label the Detection Oligo with [ $\gamma$ - $^{32}$ P]-ATP and remove unincorporated isotope.

1. Thaw frozen reagents on ice, mix thoroughly followed by a brief spin, and then place on ice.
2. Prepare [ $^{32}$ P]-labeled Detection Oligo by combining the following components at room temperature:

Components	Volume ( $\mu$ l)	Component Cap Color
Detection Oligo	2 $\mu$ l	Blue
RNase-Free Water	12 $\mu$ l	White
10X OptiKinase™ Reaction Buffer	2 $\mu$ l	Blue
[ $\gamma$ - $^{32}$ P]-ATP (6000 Ci/mmol, 150mCi/ml)	2 $\mu$ l	not supplied
OptiKinase™	2 $\mu$ l	Blue
<b>Total volume</b>	<b>20 <math>\mu</math>l</b>	

**Note:** Replace Detection Oligo with markers such as Low Molecular Weight Marker (PN 76410) when preparing radiolabeled markers.

3. Mix thoroughly followed by a brief spin in a microcentrifuge. Incubate for 30-60 min at 37°C.
4. While the reactions are incubating, prepare the Clean-Up Column as follows:
  - a. Centrifuge the Clean-Up Column for 30 sec at 750 x g to collect the dry resin at the bottom of the column.
  - b. Hydrate the resin by adding 600  $\mu$ l RNase-Free Water and vortex. Remove air bubbles by vortexing or tapping the column. Incubate at least 30 min at room temperature.  
**Note:** The Clean-Up Column can be hydrated overnight at 4°C.
  - c. Resuspend the settled resin by inverting the column several times. Ensure that no air bubbles are visible. Remove the bottom plug and place in a 2.0 ml collection tube.
  - d. Centrifuge for 2 min at 750 x g to remove the remaining water. Discard the flow-through.
5. After 30-60 min incubation, dilute the labeling reactions to 100  $\mu$ l by adding 80  $\mu$ l of RNase-Free Water.
6. Place the column from Step 4d in a clean 1.5 ml microcentrifuge tube. Without disturbing the gel bed, carefully apply the diluted sample directly onto the top of the gel bed.
7. After loading the sample, centrifuge the column for 4-6 min at 750 x g. Discard the used column in a radioactive waste container.
8. The radiolabeled Detection Oligo and markers are now ready to use.

**Note:** Store at -20°C if not required immediately. Keep on ice when in use.

### B. miRNA Capture, Ligation and Clean-Up

Once the Detection Oligo is radiolabeled, proceed to the miRNA detection assay.

#### Notes:

- The Bridge Oligonucleotide should be diluted to 0.1 pmol/ $\mu$ l with the provided 10X Capture Buffer. See Supplementary Information on Bridge Oligonucleotide (not included) Design and Preparation on page 12.
- Capture Buffer is required for detection of the Positive Control. For convenience, the Bridge Oligonucleotide in 10X Capture Buffer can be used without affecting detection specificity of the provided Positive Control.

1. Thaw frozen reagents on ice, mix thoroughly followed by a brief spin, and then place on ice.
2. Assemble the capture reaction on ice according to the table below:

Components	Positive Control	No RNA Control	Sample	Component Cap Color
Total RNA Sample	0 $\mu$ l	0 $\mu$ l	up to 8 $\mu$ l	not supplied
Positive Control	1 $\mu$ l	0 $\mu$ l	0 $\mu$ l	Red
<b>Adjust to 8 <math>\mu</math>l with RNase-Free Water</b>				White
0.1 pmol/ $\mu$ l Bridge Oligo in				
10X Capture Buffer	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	not supplied
Radiolabeled Detection Oligo	1 $\mu$ l	1 $\mu$ l	1 $\mu$ l	N/A
<b>Total volume</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	

#### Notes:

- Make a Bridge Oligonucleotide-Detection Oligo Master Mix for assay setup. Per sample combine: 1  $\mu$ l Bridge Oligonucleotide in 10X Capture Buffer + 1  $\mu$ l radiolabeled Detection Oligo.
  - Dilute all test samples to 8  $\mu$ l with RNase-Free Water then add 2  $\mu$ l of the Bridge Oligonucleotide-Detection Oligo Master Mix.
  - It is highly recommended to incubate the reactions in steps 3, 5 and 7 in a thermal cycler.
3. Mix thoroughly followed by a brief spin in a microcentrifuge. Incubate the mixture at 94°C for 1 min, 65°C for 2 min and 37°C for 10 min.
  4. Add 5  $\mu$ l of 3X Ligate-IT™ Premix (red cap) to each reaction.
  5. Mix thoroughly followed by a brief spin. Incubate for 1 hr at 30°C.  
**Optional:** If not proceeding to the next step immediately, inactivate the reaction by incubation for 10 min at 75°C and store at -20°C for later use.
  6. Add 1  $\mu$ l of Clean-Up Mix (red cap) to each reaction.

- Mix thoroughly followed by a brief spin. Incubate for 15 min at 37°C.  
**Optional:** If not proceeding to the next step immediately, inactivate the reaction by incubation for 10 min at 75°C and store at -20°C for later use.
- The ligated miRNA is now ready for gel electrophoresis.

### C. Electrophoretic Analysis

- Prepare a 12% or 15% UREA-polyacrylamide gel with 1X running buffer. (See Supplementary Information on page 14, Preparation of UREA-polyacrylamide gel.)
- Pre-run and warm the gel for 20-30 minutes.
- Transfer an aliquot of the reaction to a new tube. Add an equal volume of Gel Loading Dye (white cap).
- Transfer an aliquot of the [<sup>32</sup>P]-labeled Low Molecular Weight Marker to a new tube. Add an equal volume of Gel Loading Dye (white cap).
- Mix thoroughly followed by a brief spin in a microcentrifuge. Incubate for 3 min at 95°C. Immediately cool on ice.
- Thoroughly flush wells of the gel to remove acrylamide debris, urea and air bubbles.
- Load 2-15 µl and separate the denatured reactions. Include the [<sup>32</sup>P]-labeled markers on each gel.

#### Notes:

- The expected size of ligated miRNA is 34-37 nucleotides: 20-23 nucleotides of mature miRNA sequence plus 14 nucleotides of the labeled Detection Oligo.
  - For the radiolabeled markers prepared from 2 µl of Low Molecular Weight Marker (PN 76410) using the recommended protocol on Page 8, we suggest using 5–10 µl of a 1:50 dilution per lane for detection after 2–4 hr exposure using an intensifying screen. The radiolabeled markers can be stored at -20°C and used up to 2 months, although it is necessary to adjust the volume of markers needed per lane due to decay of the radioisotope.
  - In a 12%–15% gel, the 34-37 nucleotide labeled RNAs run between the xylene cyanol and the bromophenol blue bands. The approximate band position of the bromophenol blue band (the fast-migrating dye) is 10 nucleotides.
  - For 13 cm x 15 cm gel, run at 20-30 mA and stop when the bromophenol blue dye front has migrated to the bottom of the gel.
  - For 36 cm x 43 cm gel, run at 60 mA and stop when the bromophenol blue dye front has migrated to the middle of the gel.
- At the end of the separation, transfer the gel onto a sheet of paper, dry in a gel dryer, wrap with saran wrap and expose to a phosphorimager screen. Alternatively, transfer the gel onto a sheet of non-diffusible support material, such as processed film, wrap with saran wrap and expose to X-ray film.

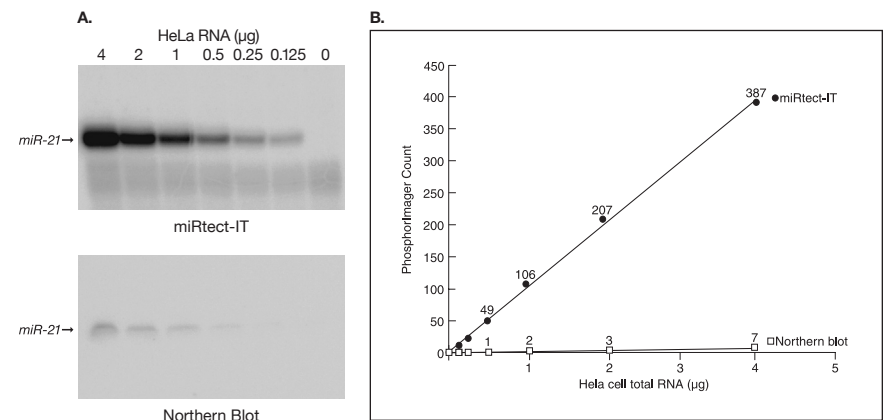
#### Notes:

- It is unnecessary to dry the gel if using X-ray film for detection. Expose the gel to X-ray film with an intensifying screen. Store for 2 hr to overnight at -80°C. The gel can be re-exposed several times if required.
- It is recommended to dry the gel if using a phosphorimager screen to prevent screen damage. Process the phosphorimager screen according to the manufacturer's instructions. The signal of the unligated labeled Detection Oligo can be very strong and therefore can become saturated. Most phosphorimaging scanners are programmed to make adjustments for the saturated bands and thus weaken the signal of the labeled miRNAs. Therefore, it may be necessary to enhance the signal intensity of the scanned image by adjusting the contrast and brightness to allow visualization of the 35-37 nt labeled miRNA.

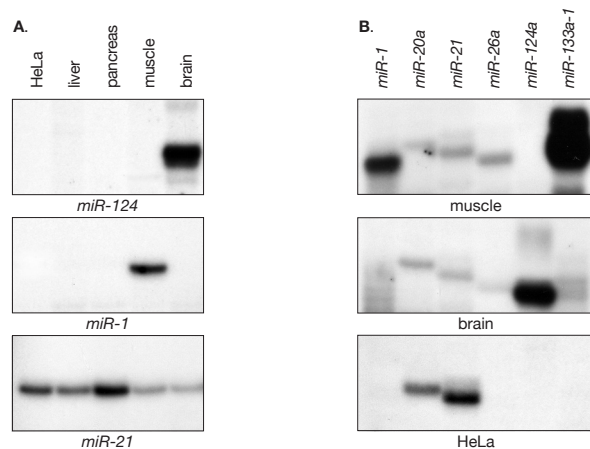
## RESULTS AND ANALYSIS

### Expected Results

Examples of results are shown in Figures 2 and 3. The expected size of the ligated miRNA is 34-37 nucleotides, which is 20-23 nucleotides of mature miRNA sequence plus 14 nucleotides of the labeled Detection Oligo. The size may be compared to a radiolabeled Low Molecular Weight Marker. The Positive Control contains a 22 nucleotide synthetic miRNA which in combination with the 14 nucleotide Detection Oligo generates a 36 nucleotide ligated fragment after separation and detection. The “No RNA Control” lane should have no signal.



**Figure 2. Detection of miR-21 by miRtect-IT™ miRNA Labeling and Detection Kit (A, upper) and Northern blot (A, lower).** Both assays were performed in parallel using HeLa cell total RNA with the indicated amounts. The image was quantitated by phosphorimager analysis (B).



**Figure 3. Expression analysis of miRNAs in human tissues (A) and tissue-specific miRNAs (B) from 250 ng total RNA by the miRtect-IT™ miRNA Labeling and Detection Kit.** The image was developed after 2 hr X-ray film exposure at -80°C.

## Target miRNA Quantification

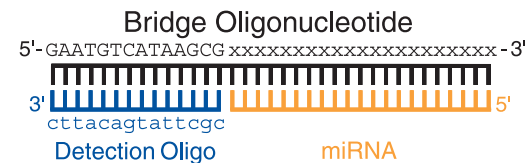
Determination of the abundance of a specific miRNA in a total RNA extract can be done by a comparison to a standard of chemically synthesized miRNA of known quantity prepared and analyzed in the same experiment. The chemically synthesized miRNA standard is the sense strand derived sequence of the mature miRNA of interest.

## SUPPLEMENTARY INFORMATION

### Bridge Oligonucleotide Design

The Bridge Oligonucleotide is a DNA oligonucleotide complementary to both the Detection Oligo and a specific miRNA at its 5' and 3' ends, respectively (Figure 4). Therefore every Bridge Oligonucleotide should have the same 14 nucleotide sequence at the 5' end, which allows a single labeling reaction of the Detection Oligo for detection of any miRNA of interest. In general, addition of unligatable-modifications to the ends of the Bridge Oligonucleotide is not always necessary. However, in some cases, it is desirable to block the 3' end or both the 5' and 3' ends of the Bridge Oligonucleotide by incorporating modification(s) such as a C3 spacer, amino modifier, inverted dT, or dideoxy-C. This ensures that unwanted side ligation reactions do not take place. USB recommends use of an unmodified Bridge Oligonucleotide as the first option for the assay.

The Bridge Oligonucleotide requires a standard desalted purification after synthesis. Further purification by HPLC or denatured PAGE is usually not necessary.



**Figure 4. Example of Bridge Oligonucleotide sequence design.**

- The 22 base *miR-21* miRNA sequence is:  
5'-uagcuuaucaagcagcugauguuga-3'
- The 14 base Detection Oligo sequence is:  
5'-CGCTTATGACATTC-3'
- The combined *miR-21* miRNA sequence and Detection Oligo sequence is:  
5'-uagcuuaucaagcagcugauguugaCGCTTATGACATTC-3'
- The reverse-complement DNA sequence is:  
5'- GAATGTCATAAGCGtcaacatcagctctgataagcta-3'
- The sequence of *miR-21* miRNA specific Bridge Oligonucleotide is:  
5'-/modification\*/-GAATGTCATAAGCGtcaacatcagctctgataagcta-/modification\*/-3'

\* Optional

### Bridge Oligonucleotide Preparation

Resuspend the Bridge Oligonucleotide with TE Buffer or RNase-Free Water to 100µM and store at -20°C. Dilute the stock solution to 100nM (0.1 pmol/µl) with 10X Capture Buffer (provided) and use 1 µl in a 10 µl assay reaction. The kit is supplied with 3 x 1 ml 10X Capture Buffer. Therefore for multiple target detection, the Bridge Oligonucleotides should be pre-diluted to 1µM with RNase-Free Water before preparing the 100nM stock solution in 10X Capture Buffer.

### General Guidelines To Prevent RNase Contamination

The following precautions are recommended to prevent RNase contamination when working with RNA:

- Wear gloves at all times while handling reagents, materials and equipment to prevent RNase contamination from hands. Change gloves after touching non-RNase free surfaces.
- Avoid using equipment and work areas that have been exposed to RNases. Clean the equipment and work surfaces with ethanol or commercially available RNase decontamination solutions.
- Clean the interior and exterior of micropipette shafts with ethanol or commercially available RNase decontamination solutions and use barrier tips.
- Use RNase-free plasticware and RNase-free buffers and reagents.

## General Considerations for Total RNA Preparation

Prepare total RNA using guanidine isothiocyanate and phenol:chloroform according to standard total RNA isolation protocols<sup>(8)</sup> with the exception that an inert carrier such as glycogen (PN 77534) or linear polyacrylamide is added to each sample. We recommend adding 20 µg of glycogen per 1 ml during alcohol precipitation to increase the recovery of small RNAs.

Samples can also be prepared by commercially available column-based methods for miRNA isolation.

Dilute RNA sample in TE Buffer (PN 75893) or RNase-Free Water (PN 71783). The purified RNA should be kept at -80°C. Avoid leaving RNA at room temperature or 4°C and multiple freezing-thawing cycles after isolation.

## Preparation of UREA-Polyacrylamide Gel

One 13 cm x 15 cm x 0.75 mm gel requires 15 ml of gel solution and one 36 cm x 43 cm x 0.8 mm gel requires 120 ml of gel solution.

### For sequencing gel system or tall gel apparatus

	12% gel	15% gel	12% gel	15% gel
Final volume	15 ml	15 ml	120 ml	120 ml
Component	Amount			
Urea (7M) (PN 75826)	7.2 g	7.2 g	57.6 g	57.6 g
40% Acrylamide/Bis Solution (19:1) (PN 75848)	4.5 ml	5.6 ml	36.0 ml	44.8 ml
5X TBE Buffer (PN 75891)	3.0 ml	3.0 ml	24.0 ml	24.0 ml

Adjust to the final volume with nuclease-free Water (PN 71783).

Stir and warm solution at 40-50°C to dissolve urea.

Cool the mixture to room temperature.

Add the following reagents immediately before pouring the gel:

TEMED (PN 76320)	7.5 µl	7.5 µl	60 µl	60 µl
10% APS (PN 76322) in nuclease-free Water	75 µl	75 µl	600 µl	600 µl

Allow to polymerize at room temperature for at least 30 min to 1 hr.

TEMED: N,N,N',N'-Tetramethylethylenediamine APS: Ammonium Persulfate  
5X TBE Buffer: Tris-Borate-EDTA Buffer (0.445M Tris, 0.445M boric acid and 0.01M EDTA)

### 10X TBE Buffer (per liter)

Component	Amount
Tris (PN 75825)	108.0 gm
EDTA (PN 15701)	5.8 gm
Boric Acid (PN 76324)	55.0 gm

Add deionized water to a final volume of 1 liter. Prepare 1X TBE Running Buffer by diluting with deionized water.

**Note:** Due to the high glycerol content of the assay components, we recommend using Glycerol Tolerant Gel (GTG) Buffer (PN 75827) in place of TBE Buffer when loading  $\geq$  half of the reaction volume on a gel. The Glycerol Tolerant Gel Buffer is specially formulated to resolve the problem of gel distortion associated with samples that contain high levels of glycerol.

### For mini gel system

	16% gel
Final volume	20 ml
Component	Amount
Urea (7M) (PN 75826)	8.4 g
30% Acrylamide/Bis Solution (29:1)	11 ml
20X Glycerol Tolerant Gel (GTG) Buffer (PN 75827)	1 ml
Adjust to the final volume with nuclease-free Water (PN 71783).	
Stir and warm solution at 40-50°C to dissolve urea.	
Cool the mixture to room temperature.	
Add the following reagents immediately before pouring the gel:	
TEMED (PN 76320)	10 µl
10% APS (PN 76322) in nuclease-free Water	100 µl
Allow to polymerize at room temperature for at least 30 min to 1 hr.	
Run in 1X GTG Running Buffer (diluted with deionized water)	

## TROUBLESHOOTING

### Problem Possible Causes and Solutions

#### Bands of unexpected size

##### 1. RNA sample is not clean.

- Repeat purification by phenol-chloroform extraction and alcohol precipitation to increase purity of the RNA sample.
- Purify the RNA sample with columns for RNA isolation (PrepEase<sup>®</sup> RNA Spin Kit, PN 78765). Adjust the amount of ethanol and sodium acetate (NaOAc) to the RNA samples to obtain a final concentration of 70% and 0.2M respectively. Load the mixture onto a column, and follow the recommended protocol for centrifugation, wash, and elution.

##### 2. RNA sample contains degraded RNA or DNA.

- Avoid RNase contamination. See Supplementary Information on General Guidelines to Prevent RNase Contamination.
- Add RNase Inhibitor (PN 71570) to the RNA sample.
- Remove DNA with rDNase I (PN 78311).
- Repeat the assay with a different batch of RNA.

##### 3. miRNA of interest has high sequence homology to non-target RNA in the sample.

- RNA with high sequence homology to the target miRNA such as its precursor or degraded RNA fragments can be base-paired with the Bridge Oligonucleotide and ligated to the Detection Oligo. Carefully analyze the size of detected bands by comparing to the labeled markers, Positive Control and No RNA Negative Control. The band corresponding to the labeled miRNA should be the size of mature miRNA plus the 14 nucleotide Detection Oligo and should not be present in the No RNA

Negative Control sample. The size of the provided Positive Control is 36 nucleotides.

- Incubate the ligation reaction at 37°C.

#### **4. Problem with Bridge Oligonucleotide.**

- Too high concentration of Bridge Oligonucleotide may cause non-specific ligation. Measure the concentration of the Bridge Oligonucleotide by  $A_{260}$  and use 0.1 pmol per assay reaction. See Supplementary Information on Bridge Oligonucleotide Preparation.
- Run a “No Bridge Negative Control” by substituting the Bridge Oligonucleotide with RNase-Free Water to assess nonspecific background signal caused by Bridge Oligonucleotide impurity.
- Incorporate modifications to the 3' end or to the 5' and 3' ends of the Bridge Oligonucleotide to block its ligation.
- Purify Bridge Oligonucleotide by denaturing PAGE and/or phenol-chloroform extraction.

#### **5. Ligase reaction is not terminated after 1 hr.**

- The components and protocol have been optimized for specific detection of a target miRNA. Carry out the assay according to the instructions.

#### **6. Clean-Up Mix reaction is incomplete.**

- Mix the reaction thoroughly after adding the Clean-Up Mix.
- Do not leave the Clean-Up Mix outside the recommended storage temperature for a long period of time. Take out an aliquot sufficient for each experiment and immediately return the unused material to -20°C.

#### ***Smear or laddered bands***

##### **1. Poor gel quality.**

- Allow gel polymerization for 1 hr or longer and pre-run the gel for 30 min.
- Thoroughly flush the loading wells with running buffer to remove acrylamide debris, urea, and air bubbles before loading.

##### **2. Overloaded samples.**

- Sample overloads may affect the separation on the gel and reduce resolution. Reduce the volume of loading sample per well.
- Use Glycerol Tolerant Gel (GTG) Buffer (PN 75827) instead of TBE Buffer (PN 75891) when loading  $\geq$  half of the reaction volume on a gel to resolve the problem of gel distortion associated with samples that contain high levels of glycerol.

#### ***Strong signal of the expected size***

##### **1. High expression of target miRNA in the sample.**

- Decrease the exposure time.
- Decrease the amount of sample used for UREA-PAGE analysis.
- Use less total RNA per assay reaction.

#### ***Weak signal of the expected size***

##### **1. Low expression of target miRNA in the sample.**

- Include the Positive Control to ensure that the assay has been performed properly and that the weak signal represents low expression of target miRNA in the sample.
- Increase the exposure time.
- Increase the amount of sample used for UREA-PAGE analysis.
- Use more total RNA per assay reaction.

##### **2. Poorly labeled Detection Oligo.**

- Improper storage and handling may cause radiolytic degradation. Prepare new radiolabeled Detection Oligo using fresh [ $\gamma$ - $^{32}$ P]-ATP and use within a few days. Store the prepared Detection Oligo at -20°C and avoid multiple freeze-thaw cycles.

##### **3. Instrument setup.**

- Enhance the signal intensity of the scanned image by adjusting the contrast and brightness to allow visualization of the 35-37 nt labeled miRNA.

#### ***No band of the expected size***

##### **1. No target miRNA present in the sample.**

- Include the Positive Control to ensure that the assay has been properly carried out and that the absence of signal is the result of no target miRNA present in the sample.
- Detect miRNAs known to be expressed in the tested sample to ensure that the absence of signal is the result of no target miRNA present in the sample, not the result of poor sample quality.

##### **2. Problem with Bridge Oligonucleotide.**

- Too low a concentration of Bridge Oligonucleotide decreases detection sensitivity. Carefully measure the concentration of the Bridge Oligonucleotide by  $A_{260}$  and use 0.1 pmol per assay reaction. See Supplementary Information on Bridge Oligonucleotide Preparation.
- Check the sequence design and synthesis of the Bridge Oligonucleotide.
- Dilute the working stock solution of Bridge Oligonucleotide with 10X Capture Buffer.

##### **3. Detection Oligo is not radiolabeled.**

- The labeling reaction failed. Successful incorporation of the isotope can be monitored by the presence of the radioactivity in the eluate after column purification. Prepare new radiolabeled Detection Oligo.

#### ***No signal detected in Positive Control reaction***

##### **1. Improper storage and handling of components.**

- The Positive Control was functionally tested using the provided reagents and protocol. Improper storage and handling may result in enzyme inactivation. Handle the components and carry out the assay according to the instructions.

- Capture Buffer is required for the reaction. The 0.1 pmol/μl Bridge Oligonucleotide in 10X Capture Buffer can be used without affecting the specific detection of the provided Positive Control.

## 2. Detection Oligo is not radiolabeled.

- The labeling reaction failed. Successful incorporation of the isotope can be monitored by the presence of the radioactivity in the eluate after column purification. Prepare new radiolabeled Detection Oligo.

If problems persist please contact USB Technical Support for assistance at (800) 321-9322 or techsupport@usbweb.com. For technical support outside the U.S., please visit our website for up-to-date contact information on the USB product distributor within your area.

## RELATED PRODUCTS

Product	Application	Pack size	Product number
Ammonium Persulfate	Gel electrophoresis	10 gm 100 gm 1 kg	76322
Boric Acid	Gel electrophoresis	500 gm 1 kg	76324
10X Capture Buffer	For use with miRtect-IT™ Kit	10 ml 50 ml	76407
rDNase I, RNase-Free	RNA purification	1000 units 2500 units	78311
Glycerol Tolerant Gel (GTG) Buffer, 20X Solution	Gel electrophoresis	1 L	75827
Glycogen, 20 mg/ml	RNA purification	1 ml 5 x 1 ml	77534
Guanidine Hydrochloride	RNA purification	100 gm 500 gm	75823
Guanidine Thiocyanate	RNA purification	100 gm 250 gm 1 kg	75818
Low Molecular Weight Marker, 10-100 nt	Gel electrophoresis	100 μl	76410
Phenol, pH 4.5, Equilibrated	RNA purification	100 ml 400 ml	77510
Phenol:Chloroform:Isoamyl Alcohol (25:24:1)	RNA purification	100 ml 400 ml	75831
PrepEase® RNA Spin Kit	RNA purification	10 preps 50 preps	78765 78766
RapidGel 40% Liquid Acrylamide Stock Solution	Gel electrophoresis	500 ml	75848
RNase Inhibitor (Human Placenta)	RNA applications	5000 units	71570
RNase Inhibitor (Recombinant)	RNA applications	5000 units	71571
TBE Buffer, 5X Solution	Gel electrophoresis	1 L 5 L	75891

Product	Application	Pack size	Product number
TE Buffer, 1X Solution	RNA purification	10 x 1 ml 100 ml 500 ml	75893
N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	Gel electrophoresis	100 gm 500 gm	76320
Tris	Gel electrophoresis	500 gm 1 kg 5 kg 10 kg	75825
Urea	Gel electrophoresis	1 kg 5 kg	75826
Water, RNase-Free	Buffer preparation RNA applications	10 x 1 ml 100 ml 500 ml 1 L 5 L	71783
Water, RNase-Free, DEPC Treated	Buffer preparation RNA applications	25 ml 10 x 1 ml 100 ml 500 ml 1 L	70783

## REFERENCES

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2. Bartel, D. P. (2004) *Cell* **116**, 281-297.
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4. Maroney, P. A., Chamnongpol, S., Souret, F. and Nilsen, T. W. (2007), *RNA*, in press.
5. Valoczi, A., Hornyik, C., Varga, N., Burgyan, J., Kauppinen, S. and Havelda, Z. (2004) *Nucleic Acids Res.* **32**, e175.
6. Moore, M. J. and Query, C. C. (2000) *Methods Enzymol.* **317**, 109-123.
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8. Sambrook, J. and Russell, D. W. (2001) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, 7.4.

## USB CORPORATION

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Please visit the USB website at [www.usbweb.com](http://www.usbweb.com) for up-to-date contact information within your area.

## Material Safety Data Sheet

Revision: 4/10/2007

Hazard information is provided for compliance with both the UK Chemicals (Hazard Information and Packaging) (CHIP) Regulations and the US Hazard Communication Standard (HCS)

### IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND COMPANY

#### SUPPLIER:

USB Corporation  
26111 Miles Road, Cleveland, Ohio 44128 Phone: (216) 765-5000  
Please visit our website at [www.usbweb.com](http://www.usbweb.com) for contact information on USB product distributors within your area.

#### PRODUCT NAME:

miRtect-IT™  
miRNA Labeling and Detection Kit

#### PRODUCT CODE:

76400

#### EEC NUMBER:

None

#### EMERGENCY CONTACT:

Chemtrec: (800) 424-9300  
Outside USA & Canada: 703-527-3887

### COMPOSITION/

#### HAZARDOUS COMPONENTS

HAZARD	CAS NO.	%WT
For Component 76405: Formamide	75-12-7	95%

#### CHIP R & S PHRASES

R:61 May cause harm to the unborn child.  
S:53 Avoid exposure - Obtain special instructions before use.  
S:45 In case of accident or if you feel unwell seek medical advice immediately (show the label where possible).  
R:36/37/38 Irritating to eyes, respiratory system and skin.  
S:23 Do not breathe vapour.  
S:24/25 Avoid contact with skin and eyes.  
S:36/37 Wear suitable protective clothing and gloves.  
R:36/37/38 Irritating to eyes, respiratory system and skin.  
S:26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.  
S:36/37 Wear suitable protective clothing and gloves.

#### TLV

See  
"Regulatory Information" Section

For Components 76407 & 76408:  
Proprietary

For Components 78334 & 76404:  
Glycerol

~50%

See  
"Regulatory Information" Section

For Component 78336:

Glycerol	56-81-5	~7.9%
Magnesium Chloride, Hexahydrate	7791-18-6	~2.0%

"See Above"

"See Component 78334 Above"

For Component 76403:

Glycerol	56-81-5	~21%
Tris-HCl	1185-53-1	~2.4%

"See Above"

R:36/37/38 Irritating to eyes, respiratory system and skin.  
S:23 Do not breathe vapour.  
S:26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.  
S:36/37 Wear suitable protective clothing and gloves.

### HAZARDS IDENTIFICATION

#### CHIP:

Component 76405: Toxic to Reproduction, Category 2.  
Components 76403, 76404, 76407, 76408, 78334 and 78336: Irritant.

#### HCS:

Component 76405: Teratogen and Irritant.  
Components 76403, 76404, 76407, 76408, 78334 and 78336: Irritant.

### FIRST-AID MEASURES

EYES: Flush with water for 15 minutes. Seek medical advice if irritation persists.  
SKIN: Flush with water, then wash thoroughly with soap and water. Remove contaminated clothing and wash before reuse. Seek medical attention if irritation persists.

INHALATION: Remove the victim from exposure and move to fresh air. If breathing is difficult, give oxygen. If not breathing, give artificial respiration. Keep victim quiet and warm. Seek immediate medical attention.  
INGESTION: Drink water and seek immediate medical attention. Avoid alcoholic beverages. Never give anything by mouth to an unconscious person.

### FIRE-FIGHTING INFORMATION

Use media suitable to extinguish the supporting or surrounding fire. Wear NIOSH (or equivalent) approved self contained breathing apparatus. For small fires only: use carbon dioxide, dry powder or foam. Emits toxic fumes under fire conditions.  
Explosion Limits for Glycerol = Lower - 1.1; Upper - Not available.  
Flash point for Glycerol = 193 °C (379.4 °F); Autoignition temperature for Glycerol = 400 °C (752 °F). Flash point for Formamide = 154 °C (309 °F). Formamide decomposes at temperatures > 180 °C.

### ACCIDENTAL RELEASE MEASURES

Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved respirator. Contain the spill with an inert absorbent and place in a suitable waste container. Avoid contact of material with skin or eyes. Use adequate ventilation.

### HANDLING AND STORAGE

Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved respirator. Use adequate ventilation. Avoid contact of material with skin or eyes. Store kit at -20 °C



Formamide  
Glycerol, Tris-HCl  
and Magnesium  
Chloride

**PERSONAL PROTECTION**

Wear appropriate personal protective equipment and clothing including lab coat, safety glasses, gloves and NIOSH-approved respirator. A qualified industrial hygienist should evaluate the need for respiratory protection. Use respiratory protection approved by NIOSH (or equivalent) and appropriate to the hazard. Avoid contact of material with skin or eyes. Mechanical ventilation or local exhaust as needed to control exposure to dust, vapors or mists. Access to a safety shower and eye-wash.

**PHYSICAL AND CHEMICAL PROPERTIES**

Appearance: Kit containing vials of solutions Solubility (Water): All components are soluble  
Chemical Formula: Not applicable Specific Gravity: No data available

**STABILITY AND REACTIVITY**

Product is stable under normal conditions. Avoid excessive heat. Incompatible with isocyanates, iodine, pyridine, sulfur trioxide, strong bases, strong acids and strong oxidizing agents. Hazardous decomposition products include ammonia, hydrochloric acid vapor, hydrogen cyanide gas and carbon oxides. Hazardous polymerization will not occur. Copper, brass, lead and rubber are attacked by Formamide. For Glycerol: Contact with Sodium Hypochlorite and Hypochlorous acid may cause an explosion.

**TOXICOLOGICAL INFORMATION****EFFECTS OF OVEREXPOSURE TO COMPONENT 76405:**

EYES: Contact causes irritation.

SKIN: Contact causes irritation. May be absorbed through the skin. Symptoms may parallel ingestion.

INHALATION: Causes irritation to the respiratory tract. Symptoms may include coughing and shortness of breath. Excessive inhalation of vapor may cause symptoms that parallel ingestion. INGESTION: Chronic ingestion or excessive dosage may cause central nervous system disorders, headache, dizziness, nausea, vomiting, abdominal pain, and unconsciousness. May affect the reproductive system. May cause damage to liver & denatures proteins. Has caused embryo toxicity and birth defects in animal studies.

TARGET ORGAN(S): Central Nervous System, Liver, Kidneys, Eyes, Reproductive System and Skin.

**EFFECTS OF OVEREXPOSURE TO COMPONENTS 76403, 76404, 76407, 76408, 76334 & 76336:**

EYES: Contact may cause irritation and slight corneal injury.

SKIN: Prolonged contact may cause irritation and/or allergic reaction.

INHALATION: Material may be irritating to mucous membranes and upper respiratory tract.

INGESTION: May cause irritation to the gastrointestinal tract with nausea, vomiting and diarrhea. ADDITIONAL INFORMATION:

Only select RTECS information is provided here. Please see actual RTECS entries for complete information.

For Formamide: Reproductive effects, irritation, mutation and toxicity data listed in RTECS under LQ0525000.

Irritation data: Eye Rabbit 100 mg = Severe (1946).

Toxicity data: Oral Rat LD50 = 5577 mg/kg (1967). Inhalation Rat LC50 = >3900 ppmv/6H.

Skin Rabbit LD50 = 17 gm/kg. Toxic effects may include incontinence and ataxia.

Harmful if swallowed, inhaled or absorbed through skin. May cause congenital malformation in the fetus.

For Glycerol: Irritation, mutation, reproductive effects and toxicity data listed in RTECS under MA8050000.

Irritation data: Skin Rabbit 500 mg/24H = Mild (1986). Eye Rabbit 500 mg/24H = Mild (1986).

Toxicity data: Oral Rat LD50 = 12600 mg/kg (1945). Inhalation Rat LC50 = >570 mg/m<sup>3</sup>/1H (1970).

For Magnesium Chloride Hexahydrate: Toxicity and mutation data listed in RTECS under OM2975000.

Oral Rat LD50 = 8100 mg/kg (1969).

For Tris-HCl: RTECS = No data available.

Definition(s): RTECS = Registry of Toxic Effects of Chemical Substances.

ACGIH = American Conference of Governmental Industrial Hygienists.

NIOSH = National Institute for Occupational Safety and Health.

No information available.

Dispose of material in accordance with applicable local, state, and federal regulations.

US DOT / IATA: No applicable information.

RCRA - No applicable information.

SARA 302 - This material does not have an RQ or TPQ.

SARA 313 - This material is not reportable under 313.

EPA TSCA Section 8(b) - For Formamide, Glycerol and Tris-HCl: Chemical Inventory.

Exposure Limits - For Formamide: ACGIH TLV-TWA: 18 mg/m<sup>3</sup> (10 ppm) Skin.

NIOSH REL TO FORMAMIDE - air: 10H TWA 10 ppm (Sk).

For Glycerol: ACGIH TLV-TWA: 10 mg/m<sup>3</sup> (total particulate).

OSHA PEL TWA: 15 mg/m<sup>3</sup> (total dust).

California Proposition 65 - No applicable information.

This data sheet is based upon information believed to be reliable. The Company makes no statement or warranty as to the accuracy or completeness of the information contained herein which is offered for your consideration, investigation and verification. Any use of the information contained in this data sheet must be determined by the user to be in accordance with appropriate applicable regulations.