

Label IT[®] μ Array[®] Biotin Labeling Kits
 Product # MIR 8010, MIR 8050

Product Name	Quantity	Product No.
Label IT [®] μ Array [®] Biotin Labeling Kits	10 labeling reactions (1 μ g each)	MIR 8010
	50 labeling reactions (1 μ g each)	MIR 8050

Label IT[®] μ Array[®] Biotin Labeling Kits are supplied with sufficient reagents to perform 10 or 50 biotin labeling reactions (1 μ g each) for microarray hybridization applications. The kits facilitate direct covalent labeling of mRNA, cDNA, or crRNA for expression profiling analyses, and provide a protocol for each type of sample. The kits do not contain sample preparation or biotin detection reagents.

Please read the entire protocol carefully and proceed with the section(s) specific for the desired experiment.

Table of Contents:	Page #
1.0 DESCRIPTION	2
1.1 General Information	2
1.2 Materials Supplied	4
1.3 Materials Required but Not Supplied	4
1.4 Storage and Stability	4
1.5 Abbreviations	4
2.0 mRNA LABELING PROCEDURE	5
2.1 mRNA Isolation	5
2.2 Biotin Labeling of mRNA	5
2.3 Purification of Biotin-labeled mRNA	6
3.0 cDNA LABELING PROCEDURE	7
3.1 First Strand cDNA Synthesis	7
3.2 cDNA Purification and Quantification	7
3.3 Biotin Labeling of cDNA	7
3.4 Purification of Biotin-labeled cDNA	8
4.0 crRNA LABELING PROCEDURE	9
4.1 Double Stranded (ds) cDNA Synthesis	9
4.2 ds cDNA Purification	9
4.3 crRNA Synthesis	9
4.4 Biotin Labeling of crRNA	10
4.5 Purification of Biotin-labeled (and fragmented) crRNA	10
5.0 HYBRIDIZATION PROCEDURE	11
6.0 DETECTION PROCEDURE	12
7.0 APPLICATION NOTES	13
8.0 TROUBLESHOOTING GUIDE	14
9.0 APPENDIX	15
9.1 Preparation of Buffers and Solutions	15
9.2 General Internet Resources	16

1.0 DESCRIPTION

1.1 General Information

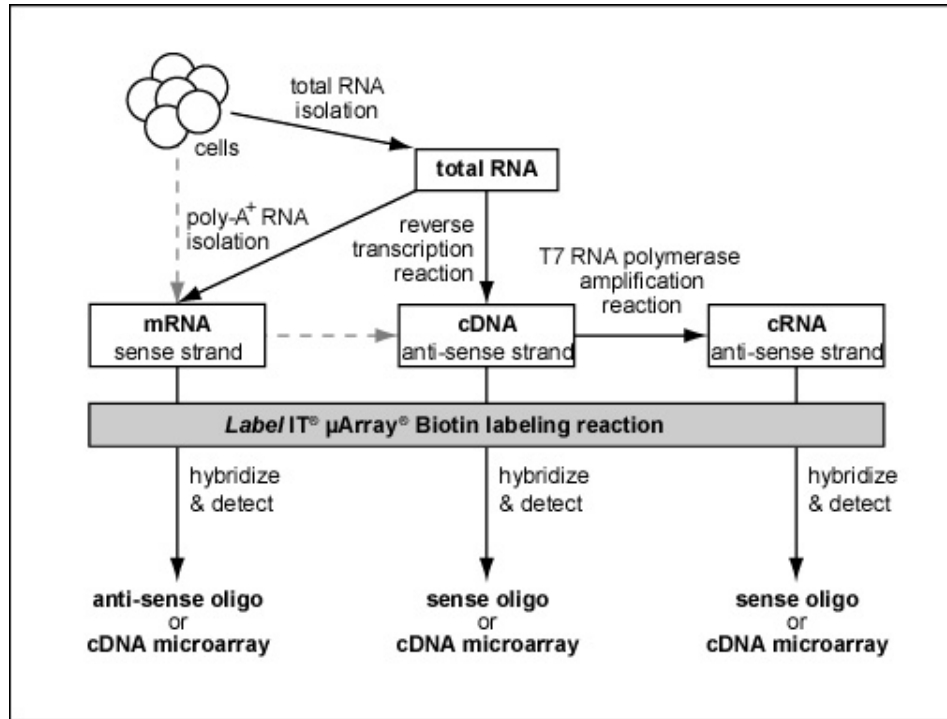
Microarrays represent an established genomics technology that allows the simultaneous hybridization of multiple target molecules on solid supports (i.e. glass slides). Expression profiling analysis, a prominent microarray application, allows the presence and relative amount of specific RNA transcripts to be measured by quantifying fluorescent signal from the microarray hybridization. For expression profiling microarray applications, samples derived from total or messenger RNA (mRNA) must be highly labeled with a marker molecule for detection. Mirus' *Label IT*[®] Nucleic Acid Labeling Reagents are designed to covalently attach marker molecules to nucleic acids in a simple one-step chemical reaction. The *Label IT*[®] Reagents directly nucleic acid bases within the DNA or RNA, and the labels do not impact hybridization performance. The ability to label DNA or RNA simply, reproducibly, and uniformly with a detectable marker represents a large technological step forward in the nucleic acid labeling field. Here, the *Label IT*[®] μ Array[®] Biotin Reagent has been optimized for the preparation of labeled nucleic acid samples for use in microarray hybridizations.

In traditional expression profiling applications, an RNA sample is enzymatically replicated (to either cDNA or cRNA) in the presence of labeled nucleotides, followed by hybridization to a microarray. Since *Label IT*[®] Reagents allow the direct chemical modification of nucleic acids, enzymatic replication and incorporation of labeled nucleotides can be eliminated from the labeling process. The *Label IT*[®] μ Array Kits allow any type of nucleic acid samples to be directly labeled— mRNA, cDNA, or cRNA (see Figure 1)- depending on experimental design.

The *Label IT*[®] μ Array[®] Biotin Labeling Kits, in direct comparison with enzymatic biotin labeling of cDNA and cRNA samples, generate more consistent and superior hybridization performance data (less variability between technical replicates and greater overall signal). Furthermore, mRNA samples that are labeled directly with the *Label IT*[®] μ Array[®] Biotin Labeling Kits do not require the traditional enzymatic replication step(s), result in sensitive hybridizations, and represent the original sample without any enzymatic replication or incorporation biases. Direct labeling of mRNA allows the detection of low copy number transcripts (less than 10 copies per cell) with the *Label IT*[®] μ Array[®] Biotin Labeling Kits. Samples labeled using these kits are compatible with hybridization on a variety of microarray surfaces, facilitating substitution into standard protocols.

The use of biotin as a marker molecule for hybridization requires a post-hybridization detection procedure with a fluorophore. As such, a biotin-labeled hybridization sample provides the flexibility to use any of a variety of established single color detection strategies. There are a variety of biotin detection reagents and amplification kits commercially available; a simple method of fluorophore conjugated streptavidin detection is provided in this protocol. Relative expression of candidate genes can be determined from independent normalized data sets for each microarray hybridization.

Figure 1. Guide to Nucleic Acid Labeling for Expression Profiling Microarray Applications



PolyA⁺ RNA is prepared directly from the specimen(s) to be analyzed or from a total RNA preparation. The polyA⁺ RNA (or mRNA) is biotin-labeled directly using the *Label IT*[®] μArray[®] Biotin Labeling Kit and hybridized to cDNA or anti-sense oligo microarrays. The RNA sample (either total or polyA⁺ RNA) can also be reverse transcribed into first-strand cDNA and then biotin-labeled using the *Label IT*[®] μArray[®] Biotin Labeling Kit. If the amount of RNA is limited, some applications may require an amplification of the sample. The RNA sample can be used to generate cRNA, which can then be biotin-labeled using the *Label IT*[®] μArray[®] Biotin Labeling Kit.

Table 1. Selecting a Sample Type for *Label IT*[®] μArray[®] Biotin Labeling Kits

Sample Type	Criteria/Features
mRNA	Allows direct hybridization of biological material No enzymatic replication bias No enzymatic incorporation bias Compatible with cDNA and anti-sense* oligo microarrays
cDNA	No enzymatic incorporation bias Compatible with cDNA and sense oligo microarrays
cRNA	Use when amplification of limited starting material is required No enzymatic incorporation bias Compatible with cDNA and sense oligo microarrays

* Currently, most oligo arrays are generated using sense-strand capture sequences, and are therefore not compatible with hybridization of labeled sense-strand RNA. Verify the design of oligo arrays before selecting a sample to label *Label IT*[®] μArray[®] Biotin Labeling Kits.

1.2 Materials Supplied

Component*	Relevant Labeling Procedure	MIR 8010 10 reactions (1 µg each)	MIR 8050 50 reactions (1 µg each)	Reagent Cap Color
<i>Label IT</i> [®] µArray [®] Biotin Reagent	mRNA, cDNA, cRNA	dried pellet	dried pellet	Brown
Reconstitution Solution	mRNA, cDNA, cRNA	40 µl	200 µl	Brown
10X Labeling Buffer M	mRNA, cDNA, cRNA	100 µl	500 µl	Purple
Reagent D1	cDNA, cRNA	150 µl	750 µl	Blue
Neutralization Buffer N1	cDNA, cRNA	190 µl	950 µl	White
0.5 M EDTA	cDNA, cRNA	500 µl	2500 µl	Green
5X Fragmentation Buffer	cRNA	250 µl	1250 µl	Orange

* Extra volume of each component is supplied to allow for slight variations in pipetting devices/usage.

NOTE: A standard biotin labeling reaction requires 1 µg of a nucleic acid sample. The *Label IT*[®] µArray[®] Biotin Labeling Reaction can be scaled up or down to label different amounts of DNA or RNA as required by alternate microarray hybridization conditions. **The labeling protocols differ for the type of sample being processed; please refer to the appropriate labeling procedure in this protocol.**

1.3 Materials Required but Not Supplied

General reagents

- MB-grade water (DNase- and RNase-free)
- RNA sample (starting material)
- Purification kit/reagents (see specific section of protocol for recommendations)

Additional reagents

Consult relevant procedural sections for specific reagents required for sample preparation, microarray hybridization, and processing.

1.4 Storage and Stability

Store the *Label IT*[®] µArray[®] Biotin Reagent at –20°C both as a dried pellet and after reconstitution. Store all other supplied reagents at 4°C or –20°C. The *Label IT*[®] µArray[®] Biotin Reagent is stable for 6 months after reconstitution. Unreconstituted *Label IT*[®] µArray[®] Biotin Reagent and all other reagents are stable for up to 1 year from the date of purchase.

1.5 Abbreviations

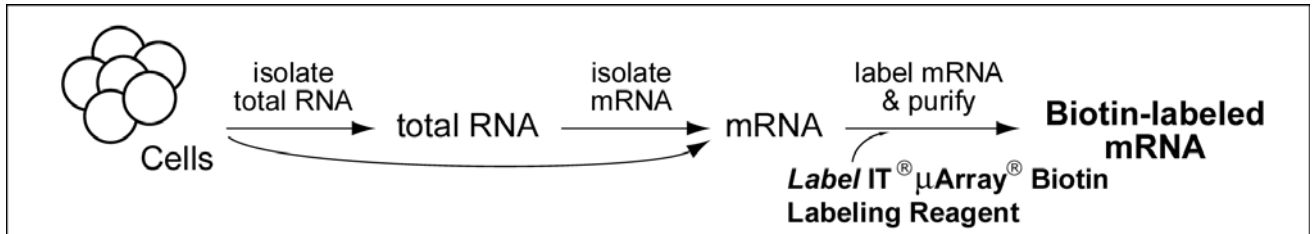
- BSA – bovine serum albumin
- SDS – sodium dodecyl sulfate (lauryl sulfate sodium salt)
- SSC – sodium chloride + sodium citrate buffer
- SSPE – sodium chloride + sodium phosphate + EDTA buffer
- SSPE-T – sodium chloride + sodium phosphate + EDTA + Triton X-100 buffer
- RT – room temperature
- MB – molecular biology

2.0 mRNA LABELING PROCEDURE

When working with RNA, wear gloves at all times. Use RNase- and DNase-free reagents, water, and plasticware. Use non-powdered gloves during all steps of sample labeling, array hybridization, array washing, detection, and scanning.

PolyA⁺ RNA isolation reagents are required but not supplied.

Figure 2. mRNA Isolation and Labeling



2.1 mRNA Isolation

NOTE: The standard application described here is for eukaryotic mRNA expression analysis. Some microarray applications may not require polyA⁺ isolation but may require isolation of alternate RNA populations, such as bacterial non-ribosomal mRNA. In these cases, Mirus recommends that the desired RNA population be isolated before proceeding with the labeling protocol. See Application Notes, Section 7.0, Part C.

1. Isolate polyA⁺ enriched RNA from sample. Direct isolation of mRNA from tissue/cells and mRNA isolation from a total RNA sample are both suitable. Mirus has successfully tested a variety of commercially available mRNA isolation kits, including PolyAtract[®] mRNA Isolation Systems (Promega Corp., www.promega.com), Poly(A)Purist[™]mRNA Purification Kit (Ambion, Inc., www.ambion.com), and Oligotex[®] mRNA Kits (Qiagen Inc., www.qiagen.com). **The mRNA must be eluted in water or dilute buffer for optimal labeling with the Label IT[®] μArray[®] Biotin Labeling Kits.** The standard elution reagents provided with the kits listed above are appropriate. Use the purification kits as recommended by the manufacturer. Prepare the amount of mRNA that is needed for the intended hybridization(s).

NOTE: Consult the manufacturer’s literature accompanying the polyA⁺ isolation kit for expected yields of polyA⁺RNA. Generally, eukaryotic total RNA consists of 1 to 4% mRNA.

2. Using a clean (50 μl) microcell cuvette, determine the absorbance at 260 nm. Use the elution buffer as the blank. Recover the RNA sample from the microcell cuvette. Quantify the mRNA using 40 μg/ml for 1 OD₂₆₀.

2.2 Biotin Labeling of mRNA

1. Warm the vial of Label IT[®] μArray[®] Biotin Reagent to room temperature and quick spin before opening. Add the indicated amount of Reconstitution Solution to the dried pellet (it may not be visible). To ensure reconstitution of the pellet, mix well by gently pipetting up and down. Four microliters of the resuspended labeling reagent will be used per one microgram of mRNA to be labeled.

<i>Label IT[®] μArray[®] Biotin Labeling Kit</i>	<i>Volume of Reconstitution Solution</i>
MIR 8010 (10 reactions, 1 μg each)	40 μl
MIR 8050 (50 reactions, 1 μg each)	200 μl

Store unused, reconstituted Label IT[®] μArray[®] Biotin Reagent tightly capped at -20°C. For subsequent use, warm the vial to RT and spin briefly before opening.

2. Prepare the labeling reaction according to the example shown. **Add the *Label IT*[®] μ Array[®] Biotin Reagent last.** For a standard 100 μ l labeling reaction with 1 μ g mRNA:

Purified mRNA sample (1 μ g)	up to 86 μ l
10X Labeling Buffer M	10 μ l
MB-grade water	bring volume to 96 μ l
<i>Label IT</i> [®] μ Array [®] Biotin Reagent	<u>4 μl</u>
Total Volume:	100 μl

NOTE: The labeling reaction may be scaled up or down, depending on the amount/volume of mRNA to be labeled. The minimal concentration of mRNA in this example is 11.6 ng/ μ l. If the mRNA sample is more dilute, simply increase the reaction volume. Alternatively, the mRNA sample can be concentrated (ethanol precipitation, lyophilization, etc.) prior to the labeling reaction.

In scaling the labeling reaction, the amount of *Label IT*[®] μ Array[®] Biotin Reagent should not constitute more than 20% of the total reaction volume. Ensure that the final concentration of Buffer M is 1X. **Use 4 μ l reagent per 1 μ g cRNA for all reaction volumes (\geq 20 μ l).**

3. Incubate the reaction at 37°C for 1 hour.

NOTE: If condensation appears at the top of the tubes during the incubation, perform a quick spin after 30 minutes of incubation. This will minimize the effect of evaporation and maintain the appropriate concentration of the reaction.

Important: The mRNA labeling reaction must NOT be treated with Reagents D1 and N1.

2.3 Purification of Biotin-labeled mRNA

1. Perform one of the following purification procedures; each is compatible with the *Label IT*[®] μ Array[®] Biotin Kit. Post-labeling purification is recommended for improved signal-to-noise and lower background levels during hybridization, though purification is not strictly required. Labeled samples may be immediately concentrated/dried down for hybridization, if desired (see Section 5.0).
 - a. Ethanol precipitation
 - i. Recommended: First add blocker/suppressor nucleic acids (e.g. species-specific Cot-1 DNA, sheared salmon DNA) required for the hybridization. This will increase the recovery of the biotin-labeled mRNA following precipitation.
 - ii. Add 0.1 volume of 5 M MB-grade NaCl and 2.5 volumes of ice cold 100% ethanol. Mix and store at -20°C (or colder) for at least 1 hour. Recommended: store precipitated sample at -20°C (or colder) until immediately before hybridization to a microarray.

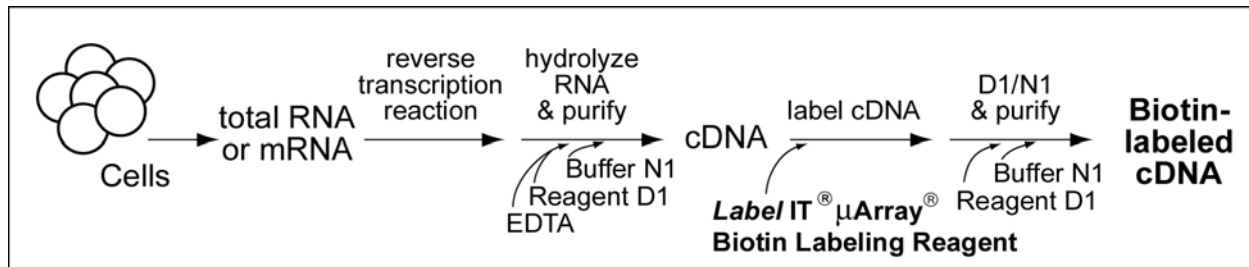
NOTE: Orient the precipitate-containing tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small RNA quantities can be invisible to the naked eye.
 - iii. Centrifuge at full speed in a refrigerated microcentrifuge at 4°C for at least 30 minutes to pellet the labeled mRNA (plus blockers). Aspirate the ethanol, being careful not to disturb the pellet.
 - iv. Gently wash the pellet once with MB-grade 70% ethanol. After an additional 15 minute centrifugation at full speed, remove all traces of ethanol with a micropipetter. Do not allow the sample to air dry extensively, as the pellet may become difficult to resuspend.
 - v. Resuspend the labeled mRNA in hybridization buffer, or buffer of choice (See Section 5.0).
 - b. Microcon Centrifugal Filter Unit (Millipore Corp., www.millipore.com) purification, using size YM-30, according to the manufacturer's directions for desalting samples.
 - c. Gel Filtration (G50) microspin column purification, according to the manufacturer's recommendations.
2. Store the purified, labeled mRNA at -80°C, or proceed directly with the microarray hybridization. See Section 5.0 for recommendations.

3.0 cDNA LABELING PROCEDURE

When working with RNA, wear gloves at all times. Use RNase- and DNase-free reagents, water, and plasticware. Use non-powdered gloves during all steps of cDNA synthesis, sample labeling, array hybridization, array washing, detection, and scanning.

Reverse transcription reagents are required but not supplied.

Figure 3. cDNA Synthesis and Labeling



3.1 First Strand cDNA Synthesis

1. Perform reverse transcription reaction(s), using either total RNA or polyA⁺ enriched RNA starting material, according to established protocols or enzyme manufacturer’s recommendations. Prepare the amount of cDNA that is needed for the intended hybridization(s).
2. Since the cDNA will be directly labeled post-synthesis, the RNA template must be removed after reverse transcription. To hydrolyze the RNA, add to the completed reverse transcription reaction(s) 0.3 volume of 0.5 M EDTA (for example, add 12 μl 0.5 M EDTA to a 40 μl reaction) and 0.1 volume of Reagent D1 (for example, add 4 μl Reagent D1 to the same 40 μl reaction).
3. Incubate at 65°C for 30 minutes and then allow the samples to slowly cool to RT.
4. Neutralize the samples by adding 0.125 original reaction volume of Neutralization Buffer N1 (for example, add 5 μl Neutralization Buffer N1 per 40 μl original reverse transcription reaction).

3.2 cDNA Purification and Quantification

1. Mirus recommends the QIAquick[®] PCR Purification Kit (Qiagen Inc.). Ensure that the pH of the sample is ~7.5 following addition of the Buffer PB. Before eluting the cDNA from the spin column, dilute the EB buffer 1/10 in MB-grade water to 1 mM Tris, pH 8.5, or use 1/10 TE buffer (1 mM Tris pH 8.0, 0.1 mM EDTA). For optimal recovery, elute the cDNA from the spin column twice with 50 μl 1/10 EB or 1/10 TE and pool eluates.
2. Using a clean (50 μl) microcell cuvette, determine the absorbance at 260 nm. Use the elution buffer as the blank. Recover the cDNA sample from the microcell cuvette. Quantify the cDNA using 37 μg/ml for 1 OD₂₆₀.

3.3 Biotin Labeling of cDNA

1. Warm the vial of *Label IT[®] μArray[®] Biotin Reagent* to room temperature and quick spin before opening. Add the indicated amount of Reconstitution Solution to the dried pellet (it may not be visible). To ensure reconstitution of the pellet, mix well by gently pipetting up and down. **Four microliters of the resuspended labeling reagent will be used per one microgram of cDNA to be labeled.**

<i>Label IT[®] μArray[®] Biotin Labeling Kit</i>	Volume of Reconstitution Solution
MIR 8010 (10 reactions, 1 μg each)	40 μl
MIR 8050 (50 reactions, 1 μg each)	200 μl

NOTE: Store unused, reconstituted *Label IT[®] μArray[®] Biotin Reagent* tightly capped at -20°C. For subsequent use, warm the vial to RT, and spin briefly before opening.

2. Prepare the labeling reaction according to the example shown. **Add *Label IT*[®] *μ*Array[®] Biotin Reagent last.** For a standard 100 μ l labeling reaction with 1 μ g cDNA:

Purified cDNA sample (1 μ g)	up to 86 μ l
10X Labeling Buffer M	10 μ l
MB-grade water	bring volume to 96 μ l
<i>Label IT</i> [®] <i>μ</i> Array [®] Biotin Reagent	<u>4 μl</u>
Total Volume	100 μl

NOTE: The labeling reaction may be scaled up or down, depending on the amount/volume of cDNA to be labeled. The minimal concentration of cDNA in this example is 11.6 ng/ μ l. If the cDNA sample is more dilute, simply increase the reaction volume. Alternatively, the cDNA sample can be concentrated (ethanol precipitation, lyophilization, etc.) prior to the labeling reaction.

In scaling the labeling reaction, the amount of *Label IT*[®] *μ*Array[®] Biotin Reagent should not constitute more than 20% of the total reaction volume. Ensure that the final concentration of Buffer M is 1X. **Use 4 μ l reagent per 1 μ g cRNA for all reaction volumes (\geq 20 μ l).**

3. Incubate the reaction at 37°C for 1 hour.
NOTE: If condensation appears at the top of the tubes during the incubation, perform a quick spin after 30 minutes of incubation. This will minimize the effect of evaporation and maintain the appropriate concentration of the reaction.
4. **Important: The cDNA labeling reaction must be treated with Reagents D1 and N1.** Add 0.1 volume of Reagent D1 (10 μ l to a 100 μ l labeling reaction), mix well, and incubate for 5 minutes at RT. Immediately add 0.1 volume of Neutralization Buffer N1 (10 μ l to the same 100 μ l labeling reaction), mix well, and incubate on ice for at least 5 minutes.

3.4 Purification of Biotin-labeled cDNA

1. Perform one of the following purification procedures; each is compatible with the *Label IT*[®] *μ*Array[®] Biotin Reagent Kit.

Post-labeling purification is recommended for improved signal-to-noise and lower background levels during hybridization, though purification is not strictly required. Labeled samples may be immediately concentrated/dried down for hybridization, if desired (see Section 5.0).

- a. Ethanol precipitation
 - i. Recommended: First add blocker/suppressor nucleic acids (e.g., species-specific Cot-1 DNA, poly A⁺ DNA, sheared salmon DNA) required for the hybridization. This will also increase the recovery of biotin-labeled cDNA following precipitation.
 - ii. Add 0.1 volume of 5 M MB-grade NaCl and 2 volumes of ice cold 100% ethanol. Mix and store at –20°C (or colder) for at least 1 hour. Recommended: store precipitated sample at –20°C (or colder) until immediately before hybridization to a microarray.
NOTE: Orient the precipitate-containing tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small DNA quantities can be invisible to the naked eye.
 - iii. Centrifuge at full speed in a refrigerated microcentrifuge at 4°C for at least 10 minutes to pellet the labeled cDNA (plus blockers). Aspirate the ethanol, being careful not to disturb the pellet.
 - iv. Gently wash the pellet once with MB-grade 70% ethanol. After an additional centrifugation at full speed, remove all traces of ethanol with a micropipetter. Do not allow the sample to air dry extensively, as the pellet may become difficult to resuspend.
 - v. Resuspend the labeled cDNA pellet in hybridization buffer, or buffer of choice. See Section 5.0.
- b. Microcon Centrifugal Filter Unit (Millipore Corp.) purification, size YM-30, according to the manufacturer's directions for desalting samples.
- c. QIAquick[®] PCR Purification Kit (Qiagen Inc.), according to the manufacturer's protocol.
- d. Gel Filtration (G50) microspin column purification, according to the manufacturer's recommendations.

2. Store the purified, labeled cDNA at -20°C , or proceed directly with the microarray hybridization. See Section 5.0 for recommendations.

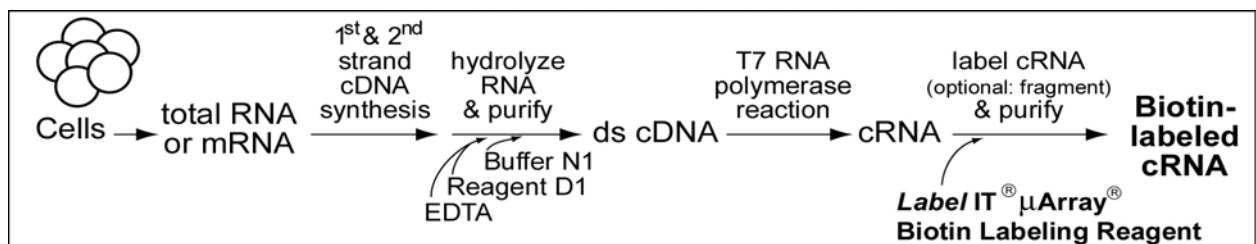
4.0 cRNA LABELING PROCEDURE

When working with RNA, wear gloves at all times. Use RNase- and DNase-free reagents, water and plasticware. Use non-powdered gloves during all steps of cRNA synthesis, sample labeling, array hybridization, array washing, detection and scanning.

Reverse transcription and T7 RNA polymerase amplification reagents are required but not supplied.

NOTE: This protocol involves an enzymatic amplification of the sample and is recommended only when starting materials may be limiting.

Figure 4. cRNA Synthesis and Labeling



4.1 Double Stranded (ds) cDNA Synthesis

1. Generate ds cDNA using either total RNA or polyA⁺ enriched RNA starting material, according to established protocols (see Section 9.2).
2. Since the cRNA will be directly labeled post-synthesis, **the RNA template must be removed**. Mirus recommends that this hydrolysis step be performed before the purification of the ds cDNA. To hydrolyze the original RNA, add to the completed second-strand cDNA reaction(s), 0.3 volume of 0.5 M EDTA (for example, add 45 μl 0.5 M EDTA to a 150 μl reaction) and 0.1 volume of Reagent D1 (for example, add 15 μl Reagent D1 to the same 150 μl reaction).
3. Incubate at 65°C for 30 minutes and then allow the samples to slowly cool to room temperature.
4. Neutralize the samples by adding 0.125 volume original reaction volume of Neutralization Buffer N1 (for example, add 18.75 μl Neutralization Buffer N1 to the 150 μl reaction). Ensure that the pH is ~ 7.5 using pH indicator strips.

4.2 ds cDNA Purification

1. Many protocols recommend phenol:chloroform extraction and ethanol precipitation to purify the ds cDNA. Although this method works well, Mirus recommends the QIAquick[®] PCR Purification Kit (Qiagen Inc.) to purify the ds cDNA from the hydrolyzed RNA (and salts).
2. Quantify the purified ds cDNA by A260 absorbance, if desired. Concentrate the ds cDNA by speed vac or lyophilization for the RNA amplification step.

4.3 cRNA Synthesis

1. Use the purified ds cDNA as template in a T7 RNA polymerase reaction, according to established protocols. Mirus routinely uses the MEGAscript[™]High Yield Transcription Kit (Ambion, Inc.). Purify the cRNA using a RNeasy[®] Mini Kit (Qiagen Inc.), according to the manufacturer's protocol for RNA cleanup, eluting the cRNA in water.
2. Using a clean RNase-free (50 μl) microcell cuvette, determine the absorbance at 260 nm. Use the elution buffer as the blank. Quantify the cRNA using 40 $\mu\text{g/ml}$ for 1 OD_{260} .

4.4 Biotin Labeling of cRNA

1. Warm the vial of *Label IT*[®] μ Array[®] Biotin Reagent to room temperature and quick spin before opening. Add the indicated amount of Reconstitution Solution to the dried pellet (it may not be visible). To ensure reconstitution of the pellet, mix well by gently pipetting up and down. **Four microliters of the resuspended labeling reagent will be used per one microgram of cRNA to be labeled.**

<i>Label IT</i> [®] μ Array [®] Biotin Labeling Kit	Volume of Reconstitution Solution
MIR 8010 (10 reactions, 1 μ g each)	40 μ l
MIR 8050 (50 reactions, 1 μ g each)	200 μ l

NOTE: Store unused, reconstituted *Label IT*[®] μ Array[®] Biotin Reagent at -20°C. For subsequent use, warm the vial to RT, and spin briefly before opening.

2. Prepare the labeling reaction according to the example shown. Add the *Label IT*[®] μ Array[®] Biotin Reagent last. For a standard 100 μ l labeling reaction with 1 μ g cRNA:

Purified cRNA sample (1 μ g)	up to 86 μ l
10X Labeling Buffer M	10 μ l
MB-grade water	bring volume to 96 μ l
<i>Label IT</i> [®] μ Array [®] Biotin Reagent	<u>4 μl</u>
Total Volume:	100 μl

NOTE: The labeling reaction may be scaled up or down, depending on the amount/volume of cRNA to be labeled. The minimal concentration of cRNA in this example is 11.6 ng/ μ l. If the cRNA sample is more dilute, simply scale up the reaction volume. Alternatively, the cRNA sample can be concentrated (ethanol precipitation, lyophilization, etc.) prior to the labeling reaction.

In scaling the labeling reaction, the amount *Label IT*[®] μ Array[®] Biotin Reagent should not constitute more than 20% of the total reaction volume. Ensure that the final concentration of Buffer M is 1X. **Use 4 μ l reagent per 1 μ g cRNA for all reaction volumes (\geq 20 μ l).**

3. Incubate the reaction at 37°C for 1 hour.

NOTE: If condensation appears at the top of the tubes during the incubation, perform a quick spin after 30 minutes of incubation. This will minimize the effect of evaporation and maintain the appropriate concentration of the reaction.
4. **Optional:** Fragmentation of the Biotin-labeled cRNA. This procedure will result in cRNA fragments less than 200 nucleotides in size, which may improve efficiency of hybridization with oligo-based capture sequences. Add 0.25 volume of 5X Fragmentation Buffer (25 μ l to a 100 μ l labeling reaction) and incubate at 94°C for 15 minutes. Place immediately on ice.

Important: The cRNA labeling reaction must NOT be treated with Reagents D1 and N1.

4.5 Purification of Biotin-labeled (and fragmented) cRNA

1. Perform one of the following purification procedures; each is compatible with the *Label IT*[®] μ Array[®] Biotin Reagent Kit.

Post-labeling purification is highly recommended for improved signal-to-noise and lower background levels following hybridization, though purification is not strictly required. Labeled samples may be immediately concentrated/dried down for hybridization, if desired (see Section 5.0).

- a. Ethanol precipitation
 - i. Recommended: First add blocker/suppressor nucleic acids (e.g., species-specific Cot-1 DNA, poly A⁺ DNA, sheared salmon DNA) required for the hybridization. This will increase the recovery of the biotin-labeled cRNA during precipitation.
 - ii. Add 0.1 volume of 5 M MB-grade NaCl and 2.5 volumes of ice cold 100% ethanol. Mix and store at -20°C (or colder) for at least 1 hour. Recommended: store precipitated sample at -20°C (or colder) until immediately before hybridization to a microarray.

NOTE: Orient the precipitate-containing tubes in the microcentrifuge in such a way that it is known where the pellet forms. Small RNA quantities can be invisible to the naked eye.
 - iii. Centrifuge at full speed in a refrigerated microcentrifuge at 4°C (plus blockers) for at least 30 minutes to pellet the labeled cRNA. Aspirate the ethanol, being careful not to disturb the pellet.
 - iv. Gently wash the pellet once with MB-grade 70% ethanol. After an additional centrifugation at full speed, remove all traces of ethanol with a micropipetter. Do not allow the sample to air dry extensively, as the pellet may become difficult to resuspend.
 - v. Resuspend the labeled cRNA pellet in hybridization buffer, or buffer of choice (see Section 5.0).
 - b. Microcon Centrifugal Filter Unit (Millipore Corp.) purification, using size YM-30 (size YM-10 if the cRNA is fragmented), according to the manufacturer's directions for desalting samples.
 - c. Gel Filtration (G50) microspin column purification according to the manufacturer's recommendations.
2. Store the purified, labeled cRNA tightly capped at -80°C or proceed directly with the microarray hybridization protocol. See Section 5.0 for recommendations.

5.0 HYBRIDIZATION PROCEDURE

Blocker/suppressor DNA (e.g. species-specific Cot-1 DNA, poly A⁺ DNA, sheared salmon sperm DNA), hybridization buffer, 20X SSC, SDS, 20X SSPE, and MES may be required but are not supplied.

1. Before hybridization, concentrate, dry down, or precipitate the biotin-labeled sample, if needed. If required, blocker/suppressor nucleic acids (e.g., species-specific Cot-1 DNA, poly A⁺ DNA, sheared salmon DNA, etc.) necessary for the hybridization can be added to the purified labeled sample prior to concentration.
2. Dilute or resuspend the biotin-labeled sample in the desired volume of hybridization buffer (see below).
3. Perform the microarray hybridization using the protocol of choice. Due to the variety of hybridization applications and formats available, general recommendations are provided. These conditions were determined to be optimal by Mirus scientists, using arrays fabricated in-house, with a variety of slide substrates. Other conditions are also compatible with samples labeled with the *Label IT*[®] *μ*Array[®] Biotin Labeling Kit, and should be optimized for the type of sample, microarray, and surface in the particular application. Please see APPENDIX Section 9.1 for the preparation of the recommended buffers.

Table 2. Standard Hybridization Conditions using cDNA (printed PCR products) Microarrays^a:

Biotin-labeled Sample	Recommended Mass per Array	Hybridization Buffer	Hybridization Temp/Duration	Post-Hybridization Washes ^b
mRNA	≥ 1.0 µg	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	50°C ~16 hours (overnight)	1. 1X SSC/0.1% SDS at 50°C, 2 x 5 min. each 2. 0.1X SSC/0.1% SDS at 50°C, 1 x 5 min. 3. 0.1X SSC, RT, 1 x 5 min. 4. Proceed immediately with detection
cDNA	≥ 1.0 µg	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	45°C ~16 hours (overnight)	1. 1X SSC/0.1% SDS at 45°C, 2 x 5 min. each 2. 0.1X SSC/0.1% SDS at 45°C, 1 x 5 min. 3. 0.1X SSC, RT, 1 x 5 min. 4. Proceed immediately with detection
cRNA (unfragmented)	≥ 1.0 µg	5X SSC, 50% formamide, 0.1% SDS with blocker/suppressor nucleic acids	45°C ~16 hours (overnight)	1. 1X SSC/0.1% SDS at 45°C, 2 x 5 min. each 2. 0.1X SSC/0.1% SDS at 45°C, 1 x 5 min. 3. 0.1X SSC, RT, 1 x 5 min. 4. Proceed immediately with detection

^a Using 22 x 40 mm coverslip area with 30 µl hybridization buffer. Hybridization volumes and masses should be scaled as appropriate for other microarray formats.

^b Perform post hybridization washes with ample volume of prewarmed buffers and moderate agitation.

Table 3. Hybridization Conditions using Oligo Microarrays^a:

Biotin-labeled Sample	Recommended Mass per Array	Hybridization Buffer	Hybridization Temp./Duration	Post-Hybridization Washes ^b
mRNA ^c				
cDNA	≥ 1.0 µg	100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween-20	45°C (50mer oligos) ~16 hours (overnight)	1. 6X SSPE, 0.01% Tween 20, 2 x 5 min. at RT 2. 100 mM MES, 0.1 M [Na+], 0.01% Tween 20, 1 x min. at 50°C 3. Proceed immediately with detection
cRNA	> 2 µg ^d	100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween-20	45°C (50mer oligos) ~16 hours (overnight)	1. 6X SSPE, 0.01% Tween 20, 2 x 5 min. at RT 2. 100 mM MES, 0.1 M [Na+], 0.01% Tween 20, 1 x 5 min. at 50°C 3. Proceed immediately with detection

^a Using 22 x 40 mm coverslip area with 30 µl hybridization buffer. Hybridization volumes and masses should be scaled as appropriate for other microarray formats.

^b Perform post hybridization washes with ample volume of prewarmed buffers and moderate agitation.

^c Currently, the majority of commercially available oligo arrays are generated using sense-strand capture sequences, and are therefore not compatible with hybridization of labeled sense-strand RNA. Anti-sense oligo arrays must be used to capture labeled sense-strand RNA samples, such as mRNA.

^d The labeling reactions may need to be scaled up for optimal hybridization performance.

6.0 DETECTION PROCEDURE

Biotin detection reagent(s) such as fluor conjugated streptavidin, BSA (ultrapure), Triton X-100, and 20X SSPE, are required but not supplied.

There are a variety of streptavidin/avidin and anti-biotin antibody fluorescent conjugates that can be used to detect hybridized biotin-labeled samples on glass slides. A general protocol using CyTM 3-conjugated streptavidin is provided that is compatible with a majority of microarray scanners:

1. After the post-hybridization washes, incubate the slide(s) in 6X SSPE-T (see APPENDIX, Section 9.1) for 5 minutes at RT.

2. Prepare sufficient Biotin Detection Solution (200 μ l per array): (see APPENDIX, Section 9.1):
 - 2 μ g/ml streptavidin-CyTM3 (Jackson ImmunoResearch Labs Inc., www.jacksonimmuno.com, or Zymed Laboratories Inc., www.zymed.com); spin before use.
 - 0.1 mg/ml BSA (Sigma-Aldrich, www.sigmaaldrich.com; Cat # A9418)
 - 6X SSPE-T
3. One at a time, remove a slide from the 6X SSPE-T wash and briefly blot edge of slide to wick off excess buffer (do not allow the slide to dry). Immediately overlay ~200 μ l Biotin Detection Solution on the array and cover with a large coverslip to evenly distribute the Biotin Detection Solution. Incubate under humidified conditions at 37°C for 20 minutes.
4. Remove Biotin Detection Solution/coverslips in 6X SSPE-T and wash three times for 5 minutes in 6X SSPE-T at RT with gentle shaking.
5. Dip the slide in water, spin or blow (compressed air) dry, and scan (532 nm laser excitation for CyTM3 detection).
6. Store hybridized and detected slides at RT, protected from light.

Important: Do not allow the slide to dry during the hybridization and detection procedure. Protect the slide from exposure to light during and following the biotin detection procedure.

7.0 APPLICATION NOTES

A. Hybridization

Due to the variety of hybridization applications and formats available, general recommendations have been provided in this protocol. Hybridization performance may require empirical optimization depending on the particular application.

B. Total RNA

Direct biotin labeling of total RNA is another attractive application of the *Label IT*[®] μ Array[®] Biotin Labeling Kits. In some applications, the hybridization of the purified labeled total RNA may provide satisfactory hybridization performance. However, Mirus recommends that the sample be enriched for labeled mRNA via polyA⁺ purification, for better hybridization sensitivity. In Section 2.0, Mirus recommends the isolation of polyA⁺ RNA prior to biotin labeling with the *Label IT*[®] μ Array[®] Biotin Labeling Kits since this strategy is more economical and provides consistently superior hybridization performance than isolating polyA⁺RNA after labeling total RNA.

C. Alternate RNA Samples

For microarray analysis applications of RNA samples other than eukaryotic mRNA, such as bacterial (or eukaryotic) ribosomal RNA, bacterial mRNA, an RNA transcribed in vitro, etc., we recommend isolating the RNA material required for the microarray hybridization and then labeling as described in the mRNA labeling protocol (Section 2.0).

D. Alternate DNA Samples

For microarray analysis of DNA samples other than cDNA (for example, genomic DNA, PCR products, etc.), we recommend isolating the alternate DNA materials required for the microarray hybridization, then labeling as described in the cDNA labeling protocol (Section 3.0).

E. Adjusting the Density of Biotin Labels

The labeling protocols in Sections 2.0, 3.0 and 4.0 have been optimized for microarray hybridization and detection performance. If there is a requirement to adjust the labeling density in the sample, increase or decrease the ratio of labeling reagent to nucleic acid in the labeling reaction. Also, the labeling density can be controlled by adjusting the incubation time; the labeling reaction is linear over the first three hours of incubation at 37°C.

F. Use of the D1 and N1 Reagents

Labeled RNA samples should not be treated with Reagents D1 and N1 (as per mRNA and cRNA labeling protocols), while DNA samples must be treated with Reagents D1 and N1 after labeling (as per cDNA protocol) to complete the labeling process.

8.0 TROUBLESHOOTING GUIDE
Poor Hybridization Signal

Problem	Solution
Suboptimal amount of sample applied to array	<ul style="list-style-type: none"> - quantify, label, and apply more sample to slide - add blockers before purifying sample by precipitation to increase yield
Poor quality RNA sample	<ul style="list-style-type: none"> - use higher quality RNA sample - prepare new cDNA or cRNA from higher quality RNA sample - use proper laboratory techniques when handling RNA sample - label and hybridize more sample to microarray
Improper detection strategy	<ul style="list-style-type: none"> - optimize detection procedure with biotin-labeled DNA spotted on glass - verify that detection instrumentation is compatible with detection reagents and fluor
Signal lost by exposure to light, environmental conditions	<ul style="list-style-type: none"> - minimize exposure to light
Poor quality array	<ul style="list-style-type: none"> - optimize array production: slide substrate, spot size, storage conditions - purchase high quality pre-spotted arrays
Poor biotin detection	<ul style="list-style-type: none"> - for cDNA and cRNA labeling, ensure that residual RNA has been hydrolyzed before labeling - ensure that the sample has been purified and quantified properly - ensure that the kit components have been stored properly - increase amount of labeling reagent in labeling reaction - increase duration of labeling reaction
Improper treatment of biotin-labeled cDNA	<ul style="list-style-type: none"> - ensure that the D1/N1 steps are performed as described
Hybridization signal 'stripped' from array	<ul style="list-style-type: none"> - decrease stringency of hybridization incubation and post-hybridization treatment/washes by increasing salt concentration and/or decreasing temperature
Suboptimal hybridization time	<ul style="list-style-type: none"> - extend hybridization time

High Background

Problem	Solution
Detection reagent remaining on slide	- centrifuge detection reagent before use - increase number and duration of post-detection washes - decrease conjugate concentration in detection protocol - increase BSA concentration in detection protocol - do not allow slides to dry out during detection protocol - do not touch slide directly or forcibly remove coverslip
Excess biotin-labeled sample applied to array	- quantify the amount of labeled material and use less in hybridization
Insufficient blocking of the array	- perform a pre-hybridization blocking step
Labeled sample not efficiently purified	- repeat purification - use alternate purification strategy
Suboptimal blocker/suppressor DNA used in the hybridization	- add more blocker and/or suppressor DNA to hybridization
Ink or marker used to identify slide	- avoid using markers or stickers to label slide - use a diamond scribe pen to label slide
Low stringency hybridization or wash conditions	- increase hybridization temperature - increase stringency of post-hybridization washes by decreasing salt concentration and/or increasing temperature
Salt from wash buffer remaining on slide	- dip rapidly several times in water before drying slide
Poor quality array	- optimize array production: slide substrate, spot size, storage conditions - purchase high quality pre-spotted arrays

9.0 APPENDIX
9.1 Preparation of Buffers and Solutions NOTE: Ensure all components are MB-grade.

Stock Solutions
20X SSC

3 M NaCl, 0.3 M sodium citrate, pH 8.0	
NaCl	175.3 g
Sodium Citrate	88.2 g
Water	800 ml
Mix well and adjust pH to 8.0 with a few drops of 10N NaOH. Adjust volume to 1000 ml with water. Sterilize by autoclaving	
Total Volume	1000 ml
Store at RT	

20X SSPE

3 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA	
NaCl	175.3 g
NaH ₂ PO ₄ ·H ₂ O	27.6 g
EDTA	7.4 g
Water	800 ml
Mix well and adjust pH to 7.4 with a few drops of 10N NaOH. Adjust volume to 1000 ml with water. Sterilize by autoclaving.	
Total Volume	1000 ml
Store at RT	

Hybridization Buffer (cDNA Microarrays)

5X SSC, 50% Formamide, 0.1% SDS	
20X SSC	250 µl
100% Formamide	500 µl
10% SDS	100 µl
Water	240 µl
Total Volume	1000 µl
Store at -20°C	

Post-Hybridization Buffers (cDNA Microarrays)

1X SSC, 0.2% SDS	
20X SSC	50 ml
10% SDS	20 ml
Water	930 ml
Total Volume	1000 ml
Store at RT	

0.1X SSC, 0.1% SDS	
20X SSC	5 ml
10% SDS	10 ml
Water	985 ml
Total Volume	1000 ml
Store at RT	

0.1X SSC	
20X SSC	5 ml
Water	995 ml
Total Volume	1000 ml
Store at RT	

Hybridization Buffer (Oligo Microarrays)	
100 mM MES, 1 M [Na+], 20 mM EDTA, 0.01% Tween 20	
12X MES Stock (see below)	83 µl
5 M NaCl	177 µl
0.5 M EDTA	40 µl
10% Tween 20	1 µl
Water	699 µl
Total Volume	1000 µl
Store at 4°C; protect from light	

12X MES Stock	
1.22 M MES, 0.89 M [Na+]:	
MES-free acid monohydrate	70.4 g
MES sodium salt	193.3 g
Water	800 ml
Mix well and adjust volume to 1000 ml. Ensure pH is between 6.5 and 6.7. Filter through a 0.2 µm filter.	
Total Volume	1000 ml
Store at 4°C; protect from light	

Post-Hybridization Buffers (Oligo Microarrays)	
6X SSPE, 0.01% Tween 20	
20X SSPE	300 ml
10% Tween 20	1 ml
Water	699 ml
Total Volume	1000 ml
Store at RT	

100 mM MES, 0.1 M [Na+], 0.05% Tween 20	
12X MES Stock	83.3 ml
5M NaCl	5.2 ml
10% Tween 20	1 ml
Water	910.5 ml
Total Volume	1000 ml
Store at 4°C; protect from light	

Biotin Wash and Detection Solutions	
6X SSPE-T	
20X SSPE	300 ml
Triton X-100	0.05 ml
Water	700 ml
Total Volume	1000 ml
Store at RT	

Biotin Detection Solution	
6X SSPE-T	200 µl
BSA (25 µg/µl)	0.8 µl
Streptavidin-Cy [™] 3 (0.8 µg/µl)	0.5 µl (spin before use)
Total Volume	201.3 µl
Use 200 µl per slide; make fresh immediately before use.	

9.2 General Internet Resources

"Anatomy of a Comparative Gene Expression Study"
<http://www.cs.wustl.edu/~jbuhler/research/array/>

Microarrays: Chipping away at the mysteries of science and medicine, from NCBI: A Science Primer
<http://www.ncbi.nlm.nih.gov/About/primer/microarrays.html>

DNA Microarray (Genome Chip) web site, by Leming Shi, Ph.D.
<http://www.gene-chips.com/>

Y. F. Leung's Functional Genomics - Microarray web site:
<http://ihome.cuhk.edu.hk/%7Eb400559/array.html>

Microarray protocols at microarrays.org:
<http://www.microarrays.org/protocols.html>

For specific questions or concerns, please contact Mirus' Technical Support at 888.530.0801 or techsupport@mirusbio.com.

For a list of citations using Mirus' products, please visit the Technical Resources section of our website (www.mirusbio.com).

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