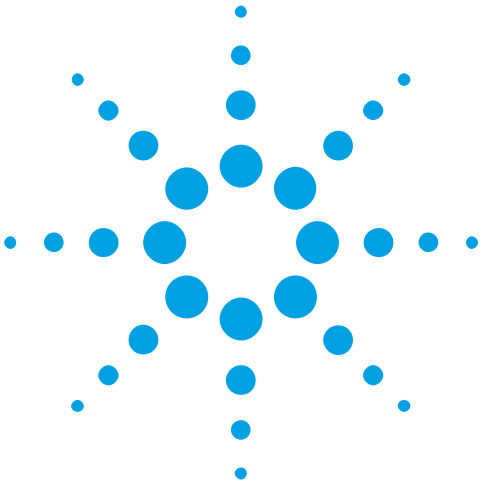
Agilent Fluorescent Direct Label Kit



Protocol

Product Number G2557A

Optimized for use with:
- Agilent cDNA Microarray Kits
- Agilent Oligo Microarray Kits

Version 2.1

June 2003

Plant RNA Quality Recommendations

See list of components or package insert for storage conditions storage conditions

Research Use Only

Agilent Technologies

Notices

Manual Part Number

G2555-98003

Edition

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www.agilent.com/chem/dnasupport

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Table of Contents

1
Introduction

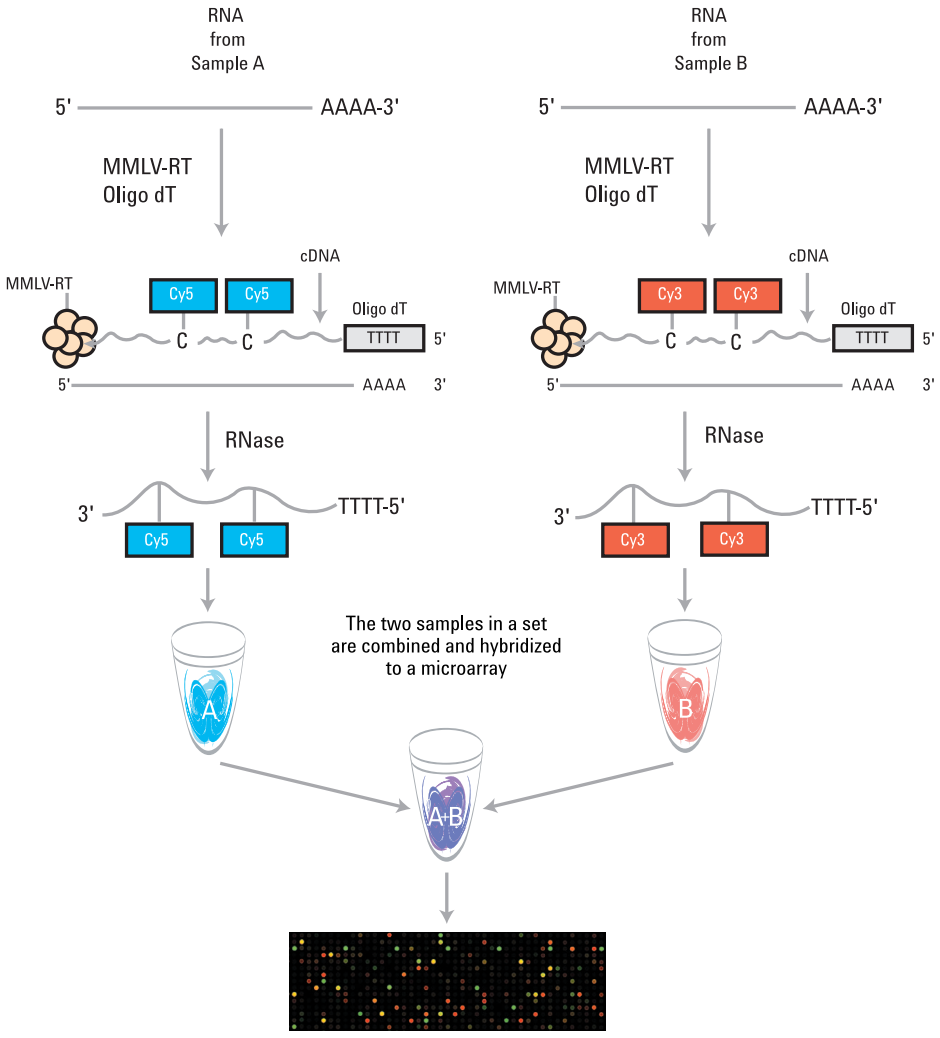
The Fluorescent Direct Label Kit generates fluorescent cDNA for use with all of Agilent's catalog cDNA microarrays and Oligo microarrays.

RNA isolated from two samples is converted to fluorescently labeled cDNA — either cyanine 3-cDNA (which is excited by a 532 nm laser), or cyanine 5-cDNA (which is excited by a 633 nm laser). Reverse transcriptase and other components facilitate the labeling reactions. A reference sample, such as a sample from a healthy subject, is labeled with one dye, and a test sample, such as a sample from a tumor, is labeled with the other dye. The resulting labeled cDNA samples are combined and hybridized to the same microarray. Genes whose expression differs between the samples are easily identifiable by scanning the microarray with a dual laser-based detection system, such as Agilent's Microarray Scanner.

The diagram below provides an overview of the procedure and shows a sample microarray image.

By using a feature extraction software package, you can link a feature to a design file and determine the relative fluorescence intensity of the two different dyes after normalization. This process allows you to quantitate differential gene expression between the two samples when you compare them.

READING THE ENTIRE PROTOCOL BEFORE YOU BEGIN IS ESSENTIAL TO SUCCESS



Workflow Time Table

Preparation of Fluorescent cDNA

cDNA Synthesis (Total time: 125 minutes)		
Step	**Temperature**	**Time**
Denature Primer & Template	70°C	10 min.
Snap Cool	Ice	5 min.
DNA Synthesis	42°C	60 min.
MMLV-RT Inactivation	70°C	15 min.
Snap Cool	Ice	5 min.
RNA Digestion	RT	30 min.
DNA Purification (Total time: 90 minutes)		
DNA Purification	RT	30 min.
SpeedVac DNA	45°C	60 min.

Kit Contents

Agilent Fluorescent Direct Label Kit (G2557A)

Component	Volume (µL)
MMLV-RT	40 µL
5x First Strand Reaction Buffer	400 µL
0.1 M DTT	200 µL
DNA Primer	40 µL
dNTP mix (-dCTP)	20µL
dCTP	10 µL
RNAse I A	40 µL

NOTE

Store all components at -20°C.

Other Required Reagents—Not Included in the Kit

- Cyanine 3-dCTP* (1.0 mM, PerkinElmer NEN catalog number NEL 576)
- Cyanine 5-dCTP* (1.0 mM, PerkinElmer NEN catalog number NEL 577)
- DNase/RNase-free distilled water (Invitrogen catalog number 10813-012)
- 100% ethanol (Amresco catalog number E193)
- QIAquick PCR Purification Kit (QIAGEN catalog number 28104)

**Catalog numbers NEL 576 and NEL 577 respectively - reference quotation number AG2001 for a 30% discount off list price. (European customers, please ask for Agilent's preferred pricing when ordering these products, as the promotional codes are not recognized in Europe.)*

Required Equipment

- Micropipettors to pipette a range of 0.1 µL to 1 mL volumes
- Sterile, nuclease-free 1.5 mL microcentrifuge tubes
- Sterile, nuclease-free aerosol barrier pipet tips
- Heating block or waterbath; set temperature to 70°C
- UV spectrophotometer and 0.1 mL volume quartz cuvettes (1 cm path length)
- Microcentrifuge
- Circulating waterbath, set temperature to 42°C
- Vortex mixer
- SpeedVac rotary dessicator
- Ice and ice bucket
- Powderless gloves
- Timer or clock
- Black, waterproof marking pen

Safety Notes

- Wear appropriate protective equipment when working in a laboratory.
- Cyanine 3-dCTP and cyanine 5-dCTP are possible carcinogens. Avoid inhalation, swallowing or contact with skin.
- Dithiothreitol (DTT) causes irritation to the skin, eyes, and respiratory tract and is harmful if swallowed or inhaled. It may also be harmful if absorbed through the skin, and may affect the central nervous system.

For the Material Safety Data Sheets (MSDS) for these compounds, please visit www.agilent.com/chem/msds

General Procedural Notes

- Follow Biosafety Level 1 (BL1) safety rules.
- Our cDNA master mix procedure specifies reagent volumes for 1 reaction and 6.5 reactions. We have added an extra half-reaction to the recipe to ensure that you do not run out of reagents in your final master mix.
- To specify reagent volumes for "n" reactions, multiply the volumes for 1 reaction by (n + 0.5).
- To prevent contamination of reagents by ribonucleases, always wear powderless laboratory gloves. Use dedicated, nuclease-free solutions, microcentrifuge tubes and pipettors with nuclease-free aerosol tips.
- Cyanine 3 and cyanine 5 are photolabile. Minimize exposure to light.
- Stock solutions that are stored in 1.5 mL microcentrifuge tubes should be prepared for use as follows:
    - Thaw the aliquot as rapidly as possible without heating it above room temperature.
    - Vortex briefly. Microcentrifuge for 5-10 seconds to drive tube contents off the tube wall and lid.
    - Store on ice until use.

NOTE

Agilent can not guarantee microarray performance using non-Agilent protocols in microarray labeling and hybridization and it does not provide support to any non-Agilent protocols.

2
Labeling Procedure

Please read the entire protocol before you begin

RNA Preparation and Qualification

It is essential that your total or poly-A^+ RNA be of high quality that meets these specifications:

1. **Size distribution (see Appendix 1 & 2 for illustrations):**
   Analyze using a denaturing gel or Agilent's 2100 bioanalyzer.

   For routine RNA QC analysis, we recommend Agilent's 2100 bioanalyzer with an RNA 6000 Nano LabChip® Kit (product number 5065-4776). The bioanalyzer provides a complete RNA profile with as little as 5 ng/µL total or poly A^+ RNA and can quickly reveal sample degradation. Where sample is limited, we recommend the RNA 6000 Pico LabChip Kit (product number 5065-4473) for qualitative measurement of RNA (200-5000 pg/µL total RNA) (500-5000 pg/µL mRNA).

   - For total RNA, ribosomal RNA should be visible at approximately 1.9 and 5 kb. The bands should be sharp and clear. If they are smeared or there are multiple peaks, then the RNA has been degraded. In addition, high molecular weight bands (>9000 kb) indicate DNA contamination in the sample.
   - For poly A^+ RNA, a faint smear in the range of 0.5 to 2 kb should be detectable.

   CAUTION

   Necessary Plant RNA Purification Steps: It is essential that you follow the recommended RNA clean-up steps in Appendix 2 when using Plant RNA. By failing to follow these RNA clean-up steps, it is likely that impure RNA will be introduced into your labeling reaction, thus affecting downstream microarray results.

2. **Determine the RNA concentration:**

   Measure UV absorbance at 260 nm using a spectrophotometer. An $\mathbf{A_{260}}$ of **1** equals an RNA concentration of approximately **40 µg/mL.**

   Plant RNA: Consult the Appendix 2 for guidance.

Required Starting Amounts of RNA Prior to Labeling

For all cDNA Microarrays and Oligo Microarrays (2 microarrays/slide format): Each labeling reaction requires either 10 µg of total RNA or 200 ng of poly A+ RNA. Store the RNA at –80°C until use.

For Oligo Microarrays (1 microarray/slide format): Each labeling reaction requires either 20 µg of total RNA or 400 ng of poly A+ RNA. Adhere to the standard direct label protocol in this publication and DO NOT double the reaction volume even though the input amount is double that of the standard labeling method. Store the RNA at -80°C until use.

Synthesis of Cyanine 3- and Cyanine 5-labeled cDNA (Time required: ~2.5 hours)

Prepare one tube of cyanine 3-labeled cDNA and one tube of cyanine 5-labeled cDNA for each microarray that you will hybridize.

1. Add sample RNA to each sterile nuclease-free reaction tube, and bring volume to 24 µL in each tube **using nuclease-free water**:

   cDNA Microarrays and Oligo Microarrays (2 microarrays/slide format) - add 10 µg of total RNA or 200 ng of poly-A+ RNA to each reaction tube.

   Oligo Microarrays (1 microarray/slide format) - add 20 µg of total RNA or 400 ng of poly-A+ RNA to each reaction tube.
2. Add 1.0 µL of DNA Primer to each reaction tube.
3. Incubate tube at 70°C for 10 minutes to denature the primer and the template RNA.
4. Place the reaction tubes on ice for 5 minutes.
5. Add 1.25 µL of either cyanine 3-dCTP (1.0 mM) or cyanine 5-dCTP (1.0 mM) to each labeling reaction. Label each tube appropriately.

NOTE

Cyanine 3 is bright pink and **cyanine 5 is bright blue**. Both are light sensitive. Minimize light exposure.

6. Immediately—prior to use, create a cDNA Master Mix from the table listed below.

   a. Gently mix the following components by pipetting **in the order indicated**, on ice.

Component	Volume (µL/rxn)	Volume (µL/6.5 rxn)
Nuclease-free Water	6.0	39.0
5x First Strand Buffer	10.0	65.0
0.1 M DTT	5.0	32.5
dNTP Mix (no dCTP)	0.5	3.3
5 mM dCTP	0.25	1.6
MMLV-RT	2.0	13.0
Total Volume =	23.75	154.4

7. Aliquot 23.75 µL of the cDNA Master Mix into each sample tube.
8. Incubate cDNA synthesis reaction at 42°C in a waterbath for 60 minutes.
9. Move the reaction tubes to a waterbath or a heating block set to 70°C. Incubate for 10 minutes to inactivate the enzyme.
10. Place the reaction tubes on ice for 5 minutes.
11. Spin samples briefly in a microfuge to drive contents off the tube wall and lid.
12. Add 1.0 µL RNAse I "A" to each reaction tube. Mix by pipetting. Incubate at room temperature for 30 minutes to degrade the RNA.

Purifying Labeled cDNA
(Time required: ~1.5 hours)

Unincorporated dye-labeled nucleotides in the hybridization solution significantly increase background fluorescence on the microarray which need to be removed from the labeling mix before proceeding to the hybridization step. We recommend using QIAGEN's QIAquick spin columns for purifying labeled cDNA samples. Please follow the QIAquick PCR Purification Kit Protocol (reproduced here) with our modifications.

NOTE

Remember to add ethanol to Buffer PE before use. (See bottle label for volume.)

13. Combine the cyanine 3- and cyanine 5-cDNA reactions for each microarray hybridization (100 µL).
14. Add 5 volumes of buffer PB (500 µL) to 1 volume of the combined cyanine 3- and cyanine 5- cDNA labeling reactions (100 µL) and mix.
15. Place a QIAquick spin column in a 2 mL collection tube.
16. To bind the DNA, apply the sample to the QIAquick column and centrifuge it in a microcentrifuge for 30 - 60 seconds at approximately 13,000 rpm (at least 10,000 x g).
17. Discard flow-through and place the QIAquick column back into the same collection tube.
18. Wash the column with 0.40 mL of QIAGEN's Buffer PE and centrifuge for 60 seconds as described in the protocol.
19. Discard flow-through, place the QIAquick column back into the same collection tube. Wash the column again with 0.40 mL of QIAGENS's Buffer PE and centrifuge for another 60 seconds.

CAUTION

Be careful not to splash the flow-through back into the tip of the column. If flow-through does come in contact with the tip, briefly spin the column and collection tube for 15 seconds prior to transfering to a new collection tube.

20. Transfer the QIAquick column from the used collection tube into a **clean** collection tube.
21. To elute the sample, add 30 µL of the QIAGEN Buffer EB (10mM Tris-Cl, pH 8.5) to the center of the column, let sit for 1 minute, then centrifuge for 60 seconds.
22. Repeat step 21 with an additional 30 µL of QIAGEN Buffer EB and elute into the same tube. The final eluted volume should be approximately 60 µL.

Concentrating Cyanine 3- or Cyanine 5-labeled cDNA
(Time required: ~1 hour)

23. Dry the solution under vacuum in a rotary dessicator until dry (approximately 60 minutes).

NOTE

To prevent cyanine dye degradation, do not use heat during drying.

24. Proceed directly to hybridization or freeze on dry ice and store at -80°C.

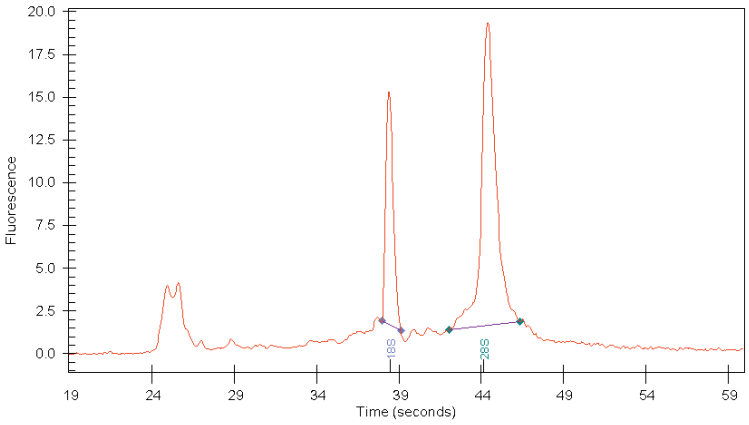
NOTE

Due to the minute amount of sample material used in this protocol, you may not see a tinted pellet after sample concentration. However, the sample is present and you may continue with hybridization.

Appendix 1
RNA Quality

Data from a high quality total RNA preparation
Although a wide variety of ribosomal peak heights and ratios exist across a broad range of RNA sample types, most high quality RNA samples share the same common features. The most distinct and intense features are the 18S and 28S ribosomal peaks (16S & 23S, prokaryotic). The baseline between 29 seconds and the 18S ribosome is relatively flat and free of small rounded peaks corresponding to smaller RNA molecules and there is an absence of smaller well-defined peaks between the two ribosomes. Any RNA migrating between the ribosomal peaks will be smooth and lack distinct peaks. Finally, depending on the RNA extraction method, the small 5S, 5.8S and tRNA may be present in the electropherogram from 24-27 seconds.

Mouse Spleen Total RNA

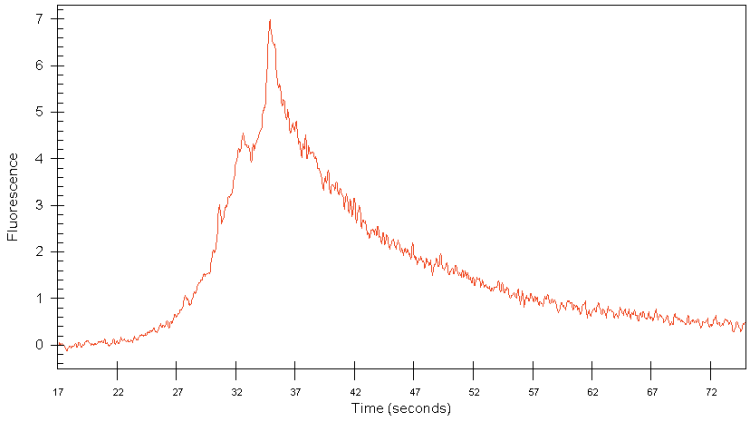


RNA Quality

Data from a high quality mRNA preparation

High quality mRNA run on the Agilent 2100 bioanalyzer typically has the shape of a broad peak, with transcripts falling in the range of 500 – 9000 bases long. The majority of transcript density falls in the size range of 1000 – 4000 bases. The electorpherograms of high quality samples are generally smooth and free of multiple large peaks. It is common for high quality mRNA samples to contain low levels of ribosomal RNA contamination, which are characterized by the presence of one to two large, well-defined ribosomal RNA peaks. The bioanalyzer software identifies and quantitates ribosomal peaks that are 5 percent of the total mRNA concentration or greater.

Bovine Brain mRNA



Appendix 2
Plant RNA Quality

Consistent with all RNA handling procedures, be careful when working with plant RNA to avoid introducing nucleases that will degrade your sample. Before labeling, be sure to check the quality and integrity of your sample via gel electrophoresis or using the Agilent 2100 bioanalyzer and RNA 6000 Nano or RNA 6000 Pico kit (www.agilent.com/chem/chip). Pictured here are examples of Arabidopsis RNA which may give you guidance when analyzing other plant RNA samples. High quality Arabidopsis RNA will have two prominent ribosomal bands accompanied by a series of smaller plastid ribosomal peaks that are isolated from the Arabidopsis chloroplasts. Notice the difference in the baseline fluorescence of the degraded Arabidopsis RNA in comparison to the high quality RNA.

Typically, purified RNA from plant and fungal samples is contaminated with high concentrations of cellulose, polysaccharides and interfering substances that are co-isolated with the RNA. Polysaccharides and secondary metabolites can interfere with the OD readings leading to errors in concentration determination. Cellulose, polysaccharides and some other plant RNA compounds will make the labeling reaction less efficient and should be removed prior to labeling.

1. It is essential that you properly prepare your plant RNA for use in labeling reactions. Available kits to isolate plant RNA include: Ambion, Inc. RNAqueous Kit, catalog number 1912 used in conjunction with the Plant RNA Isolation Aid, catalog number 9690; QIAGEN RNeasy Plant Mini Kit, catalog numbers 74903 & 74904.
2. After isolating the plant total RNA, perform an "initial RNA clean-up" step to eliminate excess plant contaminants.
    - We stongly recommend that you perform this extra clean-up step prior to isolating poly A+ RNA.
    - If you are isolating poly A+ RNA directly from plant tissues, we strongly recommend that you perform this extra clean-up step with your isolated poly A+ RNA.

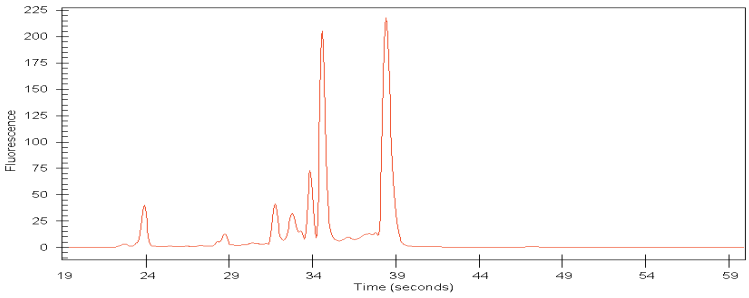
    Available kits include: QIAGEN RNeasy Kit, catalog numbers 74104 & 74106.
3. **Determining the RNA Concentration:** Measure the UV absorbance of the purified RNA sample at 230 nm, 260 nm, 280 nm, and 320 nm, either as single wavelength determinations or as part of a wavelength scan (220 nm to 320 nm) using a UV/Visible spectrophotometer. An $\mathbf{A_{260}}$ of **1** equals an RNA concentration of approximately **40 µg/mL**.
    - The ratio between the readings at 260 nm and 280 nm provides an estimate of the purity of the nucleic acid. Pure preparations of RNA (and DNA) have OD ratio values ($\mathbf{A_{260}/A_{280}}$) of between 1.8 and 2.0. Values below this indicate contamination of the sample with either phenol and/or protein. Values above this may indicate significant contamination of the sample with soluble carbohydrates.
    - OD ratio values ($\mathbf{A_{260}/A_{230}}$) less than 2.0 may indicate contamination by polysaccharides.
    - Significant absorption caused by light scattering at 320 nm indicates the presence of particulate matter in the sample.

    For reliable estimation, concentrations of at least 1 µg/mL are required.

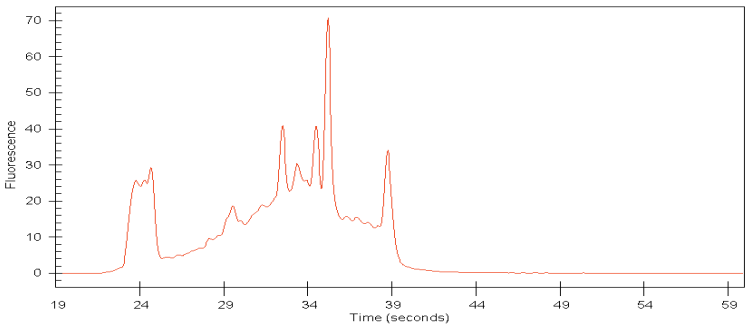
Secondary RNA Clean-up: There are instances where certain tissue or plant RNA isolation methods where the subsequent "initial clean-up" procedure may not be sufficient to remove all RNA contaminants. In these cases, we strongly recommend using **two (2) QIAGEN RNeasy clean-up columns** to ensure that all contaminants have been removed for optimal RNA quality.

Plant RNA Quality

High Quality Arabidopsis RNA



Degraded Arabidopsis RNA



Notes

Notes

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