FluoroScript™ cDNA Labeling System

For generating fluorescently labeled cDNA to use in microarray screening

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FluoroScript™ cDNA Labeling Procedure for Experienced Users

Introduction

This quick reference sheet is provided for experienced users of the FluoroScript™ cDNA Labeling System. If you are a first time user of this system, please follow the detailed protocol provided in this manual starting on page 5.

Step	Action
Prepare RNA	Isolate RNA using the method of choice. Check the quality of your RNA using agarose gel electrophoresis. You will need 25 µg total RNA or 1 µg mRNA to perform the standard labeling reaction.
Denature RNA	1. In a 0.5 ml sterile, RNase-free microcentrifuge tube combine Oligo (dT) primer and RNA as described below: $Oligo(dT)_{12-18}$ primer 1 µl RNA x µl DEPC-treated water to 5 µl 2. Incubate at 70°C for 5 minutes to denature RNA. 3. Place the tube on ice for 10 minutes. Proceed to Reverse Transcribe and Label cDNA, below.
Reverse Transcribe and Label cDNA	1. Set up the standard labeling reaction as described below using the denatured RNA. If you need to determine the first-strand synthesis yield for your sample, set up a second tube containing your sample and a control tube containing the Control RNA (see page 6 for more details). RNA Sample (from Step 3, above) 5 µl 5X FluoroScript™ Buffer 4 µl 0.1 M DTT 2 µl dNTP Mix 1 µl 1 mM fluorescent dNTP 2 µl FluoroScript™ RT 1 µl DEPC-treated water 5 µl Total Volume 20 µl 2. Incubate at 50°C for 1 hour. 3. Add 2 µl of 200 mM EDTA to stop the reaction. Proceed to Alkaline Hydrolysis and Neutralization, below.
Alkaline Hydrolysis and Neutralization	1. Add 2 µl of 1 M NaOH to the reaction tube from Step 3, above, and incubate at 70°C for 5–10 minutes. 2. Add 2 µl of 1 M HCl and vortex the tube to mix well. 3. Add 1 µl 1 M Tris pH 7.5 and vortex the tube to mix well. Proceed to Purify the Fluorescently Labeled cDNA on the next page.

Continued on next page

Step	Action
Purify the Fluorescently Labeled cDNA	1. Add 500 µl of Loading Buffer to the neutralized labeled cDNA. 2. Place a S.N.A.P.™ Column on a collection tube and load sample into the S.N.A.P.™ Column. Incubate at room temperature for 2–5 minutes. Centrifuge at 13,000 × *g* at room temperature for 60 seconds. 3. Discard the flow through. 4. Add 500 µl Wash Buffer (containing ethanol) to the S.N.A.P.™ Column and centrifuge at 13,000 × *g* at room temperature for 60 seconds. 5. Discard the flow through. 6. Place the S.N.A.P.™ Column on a new 1.7 ml microcentrifuge tube. Add 60 µl TE buffer to the S.N.A.P.™ Column and incubate at room temperature for 2–5 minutes. 7. Centrifuge at 13,000 × *g* at room temperature for 1-2 minutes. 8. Save the eluate from Step 7. 9. Repeat steps 6–8. 10. Combine the two eluates from Steps 8 and 9. The total volume should be ~100 µl. Completely dry the eluate using a SpeedVac® concentrator. Resuspend the eluate to the desired volume.

Control Reaction

We recommend performing the control labeling reaction using the Control RNA included in the system. The control reaction enables you to determine the yield of the first-strand synthesis for your sample. Please refer to page 6 to set up the control labeling reaction and page 8 to determine the yield of the first-strand synthesis.

General Information

Shipping and Storage

The FluoroScript™ cDNA Labeling System is shipped on dry ice. Upon receipt, store FluoroScript™ RT at -80°C for long-term storage or -20°C for frequent use (to minimize freezing and thawing). S.N.A.P™ columns, collection tubes, and buffers can be stored at room temperature.
The kit is stable for 6 months when stored at -20°C.

Kit Components

The table below describes the reagents provided in the FluoroScript™ cDNA Labeling System. Sufficient reagents are supplied for 20 labeling reactions, using from 25 µg up to 100 µg total RNA or 1 µg mRNA per reaction. The kit is compatible with fluorescent dyes such as cyanine 3 and cyanine 5 dUTP and dCTP, and rhodamine 110.

Item	Concentration	Amount
FluoroScript™ RT	15 U/µl in: 200 mM potassium phosphate, pH 7.1 0.1 mM EDTA 1 mM DTT 0.05% (v/v) Triton® X-100 50% (v/v) glycerol	40 µl
5X FluoroScript™ Buffer	250 mM Tris acetate, pH 8.4 375 mM potassium acetate 40 mM magnesium acetate Stabilizer	80 µl
Dithiothreitol (DTT)	0.1 M DTT in water	40 µl
dNTP Mix	Mixture of dATP, dGTP, dCTP, and dTTP in DEPC water	20 µl
Oligo(dT)$_{12-18}$	0.5 mg/ml in DEPC water	20 µl
DEPC-treated Water	—	1.25 ml
Loading Buffer	2.25 M guanidiniun HCl in 70% isopropanol	12 ml
Wash Buffer	100 mM NaCl in 75% ethanol	12 ml
S.N.A.P.™ Columns	—	20
S.N.A.P.™ Collection Tubes	—	20
Control RNA (2.3 kb)	0.5 µg/µl in DEPC water	10 µl

Product Qualification

A standard labeling reaction was set up using 1 µg of 2.3 kb control RNA, 0.5 µg oligo(dT)$_{12-18}$ primer, dNTP mix, 0.5 µCi of α-^{32}P-dCTP, 15 U/µl FluoroScript™ RT, 5X FluoroScript™ Buffer, and 0.1 M DTT. The first-strand cDNA yield after 1 hour at 50°C must be ≥ 18%. The percentage of cpm recovered following precipitation of the cDNA and S.N.A.P. purification must be at least 30%.

Overview

Introduction

The FluoroScript™ cDNA Labeling System provides a method to generate fluorescently labeled cDNA. In this direct labeling protocol, an oligo(dT) primer is annealed to the denatured RNA template and reverse transcribed in the presence of fluorescently labeled dNTP to produce fluorescently labeled cDNA. The reverse transcription reaction is catalyzed by FluoroScript™ reverse transcriptase (RT), an avian reverse transcriptase with reduced RNase H activity (see below).

The FluoroScript™ cDNA Labeling System is specifically designed to provide higher incorporation of fluorescently labeled nucleotides in your cDNA sample than other RTs.

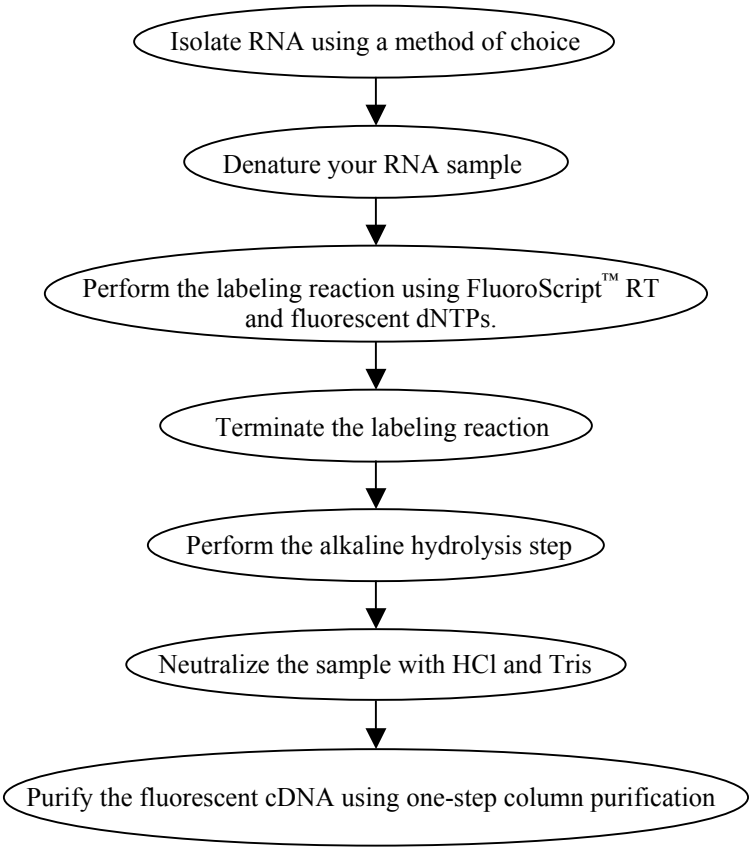
Advantages of FluoroScript™ RT

The following properties of FluoroScript™ RT make this enzyme an ideal tool to generate fluorescently labeled cDNA:

- Efficiently synthesizes fluorescently labeled cDNA from total RNA or mRNA as the enzyme is not inhibited by ribosomal and transfer RNA
- Increased thermostability of the enzyme, allowing it to be used at temperatures up to 55°C resulting in increased yields of cDNA
- Reduced RNase H activity to prevent RNA degradation

Experimental Outline

The flow chart below outlines the experimental steps of the system:



Materials Supplied by the User

You should have the following items on hand before using the FluoroScript™ cDNA Labeling System.

- 1 mM fluorescent dNTP (Cy3 or Cy5)
- Vortex mixer
- Microcentrifuge
- 70% ethanol
- 95% ethanol
- Aerosol resistant pipette tips
- Water baths
- 20 mM and 200 mM EDTA
- 1 M NaOH
- 1 M HCl
- 1 M Tris, pH 7.5
- TE buffer
- α-^{32}P-labeled dCTP or α-^{33}P-labeled dCTP
- Yeast tRNA (see page 10 for ordering information)
- Glass fiber filters (Fisher catalog no. 1822-914)
- Heat lamp
- 5% trichloroacetic acid (TCA)
- 10% TCA
- 1% sodium pyrophosphate
- Scintillation counter
- Sterile microcentrifuge tubes

Methods

Isolating RNA

Introduction

You need to isolate high-quality RNA using a method of choice prior to using the FluoroScript™ cDNA Labeling System. You may use 25–100 µg total RNA or 1 µg mRNA as your starting material. Please follow the guidelines provided below to avoid RNase contamination.

General Handling of RNA

When working with RNA:

- Use disposable, individually wrapped, sterile plasticware.
- Use aerosol resistant pipette tips for all procedures.
- Use only sterile, new pipette tips and microcentrifuge tubes.
- Wear latex gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin.
- Use proper microbiological aseptic technique when working with RNA.
- Dedicate a separate set of pipettes, buffers, and enzymes for RNA work.
- Microcentrifuge tubes can be taken from an unopened box, autoclaved, and used for all RNA work. RNase-free microcentrifuge tubes are available from several suppliers. If it is necessary to decontaminate untreated tubes, soak the tubes overnight in a 0.01% (v/v) aqueous solution of diethylpyrocarbonate (DEPC), rinse the tubes with sterile distilled water and autoclave the tubes.

You may use RNase AWAY™ Reagent, a non-toxic solution available from Invitrogen (see page on page 10 for ordering information) to remove RNase contamination from surfaces. For further information on controlling RNase contamination, see Ausubel, *et al.*, 1994, Sambrook, *et al.*, 1989.

Isolating RNA

The starting material can either be total RNA or mRNA. In general, as little as 25 µg total RNA or 1 µg mRNA is sufficient to generate fluorescently labeled cDNA using the FluoroScript™ cDNA Labeling System.

You may isolate mRNA or total RNA using a method of choice prior to using this kit. We recommend isolating mRNA using the Micro-FastTrack™ 2.0 or FastTrack® 2.0 mRNA Isolation Kits. To isolate total RNA, we recommend using the Micro-to-Midi Total RNA Purification System, Trizol® Reagent (Chirgwin *et al.*, 1979; Chomczynski and Sacchi, 1987), or the S.N.A.P.™ Total RNA Isolation Kit. Ordering information for these kits is provided on page 10.

Once you have isolated the RNA, you need to check the quality of your RNA preparation (see next page). After preparing the RNA, we recommend that you proceed directly to **Reverse Transcribing and Labeling DNA using FluoroScript™ RT** (see page 5).

Continued on next page

Checking the RNA Quality

To check RNA integrity, analyze 500 ng of your RNA by agarose/ethidium bromide gel electrophoresis. You may use a regular 1% agarose gel or a denaturing agarose gel (Ausubel *et al.*, 1994). For total RNA using a regular agarose gel you should see the 28S and 18S rRNA bands. The 28S band should be twice the intensity of the 18S band. The 28S band should run at 4.5 kb and the 18S band should run at 1.9 kb. If you do not load enough RNA, the 28S band may appear to be diffuse. If you are using a denaturing gel, the rRNA bands should be very clear and sharp. mRNA will appear as a smear from 0.5 to 12 kb.

If you do not detect any RNA, you will need to repeat RNA isolation. Be sure to follow the recommendations listed on the previous page to prevent RNase contamination. Please refer to the **Troubleshooting** section on page 9.

Reverse Transcribing and Labeling RNA

Introduction

Once you have isolated RNA and checked the quality of your RNA preparation, you are ready to reverse transcribe RNA using fluorescently labeled dNTPs to generate fluorescently labeled cDNA.

Before Starting

Have the following items on hand before starting:

- Oligo(dT)$_{12-18}$ primer (supplied with the kit)
- DEPC-treated water (supplied with the kit)
- 200 mM EDTA
- 1 M NaOH
- 1 M HCl
- 1 M Tris, pH 7.5
- Water baths set at 50°C and 70°C
- Ice
- 0.5 ml RNase-free microcentrifuge tubes
- α-^{32}P-labeled dCTP or α-^{33}P-labeled dCTP
- 1 mM fluorescent dNTP (Cy3 or Cy5)

Control RNA

Control RNA (2.3 kb) is included in the kit to help you verify the proper functioning of the system and to determine the yield of your cDNA sample. We strongly recommend performing the control reaction if you are a first time user of the FluoroScript™ cDNA Labeling System. Instructions are provided on the next page to set up the labeling reaction using the Control RNA. Calculations are provided on page 8.

Amount of RNA

The standard labeling reaction uses 25 µg total RNA or 1 µg mRNA and 1 µl of FluoroScript™ RT. If you are using RNA amounts greater than the amount used in the standard labeling reaction, please refer to the table below to determine the amount of FluoroScript™ RT to use for reverse transcription.

Total RNA	mRNA	FluoroScript™ RT
25 µg	1–2 µg	1 µl (15 U)
50 µg	—	1.5 µl (22.5 U)
100 µg	3–4 µg	2 µl (30 U)

Denaturing RNA

1. In a 0.5 ml RNase-free tube, combine the following reagents. For the Control RNA sample, use 2 µl Control RNA supplied in the kit.

Oligo(dT)$_{12-18}$ primer	1 µl
RNA	x µl
DEPC-treated water	to 5 µl

2. Incubate at 70°C for 5 min to denature RNA.
3. Place the tube on ice for 10 min.

Note: Denaturing the RNA template is optional, and you may obtain efficient reverse transcription without performing this step. However, heating the RNA prior to cDNA synthesis helps remove any secondary structure that may prevent full-length cDNA synthesis.

Continued on next page

Reverse Transcribing and Labeling RNA, Continued

Labeling Reaction

1. Vortex the 5X FluoroScript™ Buffer for 5 seconds just prior to use.
2. Set up on ice three labeling reactions using the denatured RNA (from Step 3, above):

   Sample 1: Contains your RNA sample and is used to generate the probe

   Sample 2: Contains your RNA sample and is used to determine the first-strand synthesis yield

   Control: Contains 2 µl of Control RNA provided in the kit and is used to determine the first-strand synthesis yield

Reagent	Sample 1	Sample 2	Control
RNA Sample (from Step 3, above)	5 µl	5 µl	5 µl
5X FluoroScript™ Buffer	4 µl	4 µl	4 µl
0.1 M DTT	2 µl	2 µl	2 µl
dNTP Mix	1 µl	1 µl	1 µl
α-^{32}P-labeled dCTP or α-^{33}P-labeled dCTP	—	0.5 µCi	0.5 µCi
1 mM fluorescent dNTP	2 µl	2 µl	—
FluoroScript™ RT (15 U/µl)	1 µl*	1 µl*	1 µl
DEPC-treated water	5 µl	to 20 µl	to 20 µl

* Use the appropriate amount of FluoroScript™ RT based on the amount of your RNA (see **Amount of RNA**, previous page).

3. Vortex the tubes gently to mix the contents and centrifuge the tubes briefly to collect the contents from the cap of the tube.
4. Incubate the tubes at 50° C for 1 hour.
5. Add 2 µl of 200 mM EDTA to Sample 1 to stop the reaction and proceed to **Alkaline Hydrolysis and Neutralization**, next page.
6. For Sample 2 and the Control tube, proceed to **Determining the First-Strand Synthesis Yield** on page 8. Also save 2 µl of the reaction mixture for the yield calculation.

Alkaline Hydrolysis and Neutralization

1. Add 2 µl of 1 M NaOH to the Sample 1 reaction tube from Step 5, previous page.
2. Incubate at 70° C for 10 minutes.
3. Add 2 µl of 1 M HCl and vortex the tube to mix well.
4. Add 1 µl 1 M Tris pH 7.5 and vortex the tube to mix well. Proceed to **Purifying the Fluorescently Labeled cDNA**, next page.

Purifying the Fluorescently Labeled cDNA

Introduction

After you have generated the fluorescently labeled cDNA, you need to purify the cDNA to remove excess fluorescent dNTPs. A quick one-step protocol using the S.N.A.P.™ columns included in the system is provided below. Other methods of purification are suitable.

Before Starting

Be sure to have the following items on hand before starting:

- TE Buffer
- SpeedVac® concentrator
- S.N.A.P.™ Columns and Collection Tubes (supplied with the kit)
- Loading Buffer and Wash Buffer (supplied with the kit)

One-Step Procedure

1. Add 500 µl of Loading Buffer to the neutralized labeled cDNA (Step 4, previous page). Mix well by vortexing.
2. Place a S.N.A.P.™ Column on a collection tube and load your sample on the S.N.A.P.™ Column.
3. Incubate the S.N.A.P.™ Column containing your sample at room temperature for 2–5 minutes. Centrifuge at 13,000 × *g* at room temperature for 60 seconds.
4. Discard the flow through.
5. Add 500 µl Wash Buffer to the S.N.A.P.™ Column.
6. Place the S.N.A.P.™ Column onto the collection tube and centrifuge at 13,000 × *g* at room temperature for 60 seconds.
7. Discard the flow through.
8. Place the S.N.A.P.™ Column onto a new 1.7 ml microcentrifuge tube. Add 60 µl TE buffer to the S.N.A.P.™ Column. Incubate at room temperature for 2–5 minutes.
9. Centrifuge at 13,000 × *g* at room temperature for 1–2 minutes.
10. Save the eluate from Step 9.
11. Repeat steps 8–10.
12. Combine the two eluates from Steps 10 and 11. The total volume should be ~100 µl. Completely dry the sample using a SpeedVac® concentrator. Resuspend the sample to the desired volume.

Note: You can store the concentrated purified cDNA at -20°C or -80°C **in the dark** for long-term storage.

The Next Step

After you have generated fluorescently labeled cDNA, you are ready to use the labeled cDNA in any application of choice, including glass microarray hybridization. Resuspend your probe in a suitable buffer and proceed to a hybridization method of choice.

Determining the Yield of First-Strand Synthesis

Introduction

Instructions are provided below to calculate the yield of your first-strand synthesis.

Before Starting

Be sure to have the following items on hand before starting:

- Yeast tRNA (see page 10 for ordering information)
- 20 mM EDTA
- Glass fiber filters (Fisher Catalog no. 1822-914)
- Heat lamp
- 5% and 10% trichloroacetic acid (TCA)
- 1% sodium pyrophosphate
- Scintillation counter
- Sterile microcentrifuge tubes

Procedure

1. Add 2 µl of the reaction mix from Step 6, page 6, to a microcentrifuge tube containing 43 µl of 20 mM EDTA (pH 7.5) and 5 µl of yeast tRNA (5 µg). Mix well by vortexing.
2. Spot 10 µl aliquots from each sample onto duplicate glass fiber filters.
3. Dry the filters under a heat lamp.
4. Set one filter aside. This filter will be used to determine the specific activity of dCTP in the reaction.
5. Wash the second filter once in ice cold 10% TCA containing 1% sodium pyrophosphate for 10 minutes at room temperature on a rotary shaker.
6. Wash the filter twice in 5% TCA for 10 minutes.
7. Wash the filter with 95% ethanol for 10 minutes at room temperature. This filter will be used to determine the yield of the first-strand cDNA.
8. Count both filters using a standard scintillation counter.

Calculating the Yield

Calculate first-strand synthesis yield as follows:

$$\text{Specific activity (cpm/pmol dCTP)} = \frac{\text{cpm of 10 µl from unwashed sample}}{\text{100 pmol dCTP}}$$

$$\text{Amount of cDNA (µg)} = \frac{\text{cpm of washed sample x 50 x 4 pmole dNTP/pmol dCTP}}{\text{Specific activity x 3,030 pmol dNTP/µg cDNA}}$$

$$\text{Yield} = \frac{\text{Amount of cDNA (µg) x 100}}{\text{Amount of mRNA used (µg)*}}$$

*If you are using total RNA as your starting material, the mRNA will be 1–2% of total RNA.

Expected Results

The first-strand yield for the control RNA should be at least 18%. If you do not obtain the expected yield, please refer to the **Troubleshooting** section on the next page for some tips on improving the yield.

Troubleshooting

Introduction

RNA quality is the key factor that will influence the outcome of your results using the FluoroScript™ cDNA Labeling System. The table below provides some specific troubleshooting tips.

Problem	Cause	Solution
28S and 18S bands are not observed after isolation of total RNA and agarose gel electrophoresis	Too little RNA loaded on the gel	Be sure to load at least 250 ng of RNA for analysis.
	RNA is degraded due to RNase activity	Follow the guidelines on page 3 to avoid RNase contamination. Use fresh sample for RNA isolation.
No cDNA produced	cDNA synthesis performed at a high temperature	Perform the cDNA synthesis at 50° C.
	Incorrect reaction conditions used	Verify that all reaction components are included in the reaction and use reagents provided in the system. Verify the labeling reaction conditions using the control RNA provided in the kit.
	Concentration of template RNA is too low	Increase the concentration of template RNA. Use at least 25 µg total RNA or 1 µg mRNA.
	Poor quality RNA used or RNA is degraded	Check the quality of your RNA preparation (see page 4). If RNA is degraded, use fresh RNA.
	RNase contamination	Add RNaseOUT™, an RNase inhibitor (see page 10), to prevent RNA degradation.
	RT inhibitors are present in your RNA sample	Inhibitors of RT include SDS, EDTA, guanidinium chloride, formamide, sodium phosphate and spermidine (Gerard, 1994). Remove inhibitors from your RNA sample by performing an additional 70% ethanol wash after ethanol precipitation. Test for the presence of inhibitors by mixing 1 µg of control RNA with 25 µg total RNA or 1 µg mRNA and compare the yields of first-strand synthesis.
	Improper storage of FluoroScript™ RT	Store the enzyme at -80°C for long-term storage. For daily use, store the enzyme at -20°C to minimize freezing and thawing.
	Improper procedure used for S.N.A.P.™ Column purification	Follow the procedure without any modifications.
Shorter cDNA size	Prolonged alkaline hydrolysis step	Optimize incubation time of the alkaline hydrolysis step to minimize DNA degradation.

Appendix

Accessory Products

Additional Products

Many of the reagents in the FluoroScript™ cDNA Labeling System, as well as additional reagents that may be used with this system are available separately from Invitrogen. Ordering information is provided below.

Product	Quantity	Catalog no.
RNase AWAY™ Reagent	250 ml	10328-011
Yeast tRNA	100 mg	16051-039
Micro-FastTrack™ 2.0 mRNA Isolation Kit	20 reactions	K1520-02
FastTrack® 2.0 mRNA Isolation Kit	6 reactions	K1593-02
Micro-to-Midi Total RNA Purification System	50 reactions	12183-018
Trizol® Reagent	100 ml	15596-026
S.N.A.P.™ Total RNA Isolation Kit	50 reactions	K1950-01
Oligo(dT)$_{12-18}$ Primer	25 µg	18418-012
RNaseOUT™ Recombinant Ribonuclease Inhibitor	5000 units	10777-019
Human Cot-1 DNA®	500 µg	15279-011
Mouse Cot-1 DNA®	500 µg	18440-016
Random primers	9 A_{260} units	48190-011
DEPC-treated water	4 × 1.25 ml	10813-012
10% SDS solution	4 × 100 ml	15553-027
20X SSC	1 L	15557-044
20x SSPE	1 L	15591-043
10 mM dNTPs	100 µl 1 ml	18427-013, R725-01

Purchaser Notification

Limited Use Label License No. 123: FluoroScript™ Enzyme

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