FastRNA® Pro Blue Kit

Rapid Isolation of Total RNA from Gram Positive and Gram Negative Bacteria Using the FastPrep® Instrument

Revision # 6025-050-3F24

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Application Manual

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50 Samples

Storage temperature:
Refrigerated or ambient temperature (4ºC or 15–30ºC)

DO NOT expose RNA*pro*™ Solution to light for extended periods of time. Store in the original bottle in the closed kit box.

Note:
An empty space in the box insert has been provided for convenient storage and access to the RNA*pro*™ Solution when it has been removed from the safety shipping container.

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1. Introduction to the FastRNA® Pro Blue Kit and the FastPrep® Instrument

The FastRNA® Pro Blue Kit is a single reagent extraction method designed to quickly and efficiently isolate total cellular RNA from gram positive and gram negative bacteria. The RNA*pro*™ Solution included in the kit is designed to efficiently inactivate cellular RNases during cell lysis to prevent RNA degradation. During use the RNA*pro*™ Solution is mixed with the bacterial sample in a tube containing a specifically selected lysing matrix. The tube is then processed in the FastPrep® Instrument for 40 seconds to release the total cellular RNA, DNA and proteins. Following the FastPrep® homogenization the RNA is purified and isolated by chloroform extraction and ethanol precipitation. The purified RNA is ready for downstream applications, including RT-PCR and northern analysis. The average RNA yield from 10^{10} bacteria is greater than 50 µg.

The FastPrep® Instrument is a high-speed, benchtop device that uses a proprietary vertical angular motion (1) to produce sample homogenization by simultaneous matrix impaction from multiple directions. The FastPrep® Instrument provides an extremely quick and highly reproducible homogenization that surpasses traditional lysis methods using enzyme digestion, sonication, blending, douncing and vortexing. When used with FastPrep® kits the FastPrep® Instrument permits the release and purification of intact DNA, RNA and proteins from virtually any source, including bacteria, yeast and fungi, spores, plant seeds and leaves, animal tissue, organs and blood, etc.

2. Kit Components and User Supplied Materials

2.1 FastRNA® Pro Blue Kit Components

Product Description	Qty.
RNA*pro*™ Solution	1 x 55 ml bottle
DEPC-H_2O	1 x 15 ml bottle
Lysing Matrix B	50 x 2 ml tubes
Short protocol	1 each
User manual	1 each
MSDS	1 each
Certificate of Analysis	1 each

2.2 User Supplied Materials

FastPrep® Instrument (Cat # 6001-100, -120, or -220)
Microcentrifuge
Pipettmen
RNase Erase® (Cat # 2440-204), recommended
Chloroform
100% ethanol
75% ethanol
1.5 or 2.0 ml RNase-free microcentrifuge tubes
Agarose
Gel loading dye and RNA size marker

3. Important Considerations before Use

The presence or introduction of RNase during the procedure may result in sample degradation. It is strongly recommended that the user minimize the potential for RNase contamination by using gloves throughout the procedure, using DEPC-H_2O and by treating pipettmen, work area, gel box and gel comb with RNase Erase®. Additional RNA handling methods and precautions may be found in references 2 and 3.

The volume after the addition of RNA*pro*™ Solution to the sample has been calculated to maintain a sufficient air space in the sample tube during FastPrep® Instrument processing. Sample loss or tube failure may result from overfilling the matrix tube. The matrix tube caps must be secure, but not over-tightened, to prevent sample leakage. If the sample is too large for processing in a single tube, divide the sample and process using multiple tubes.

Confirm the sample tubes spin freely and will not scrape the microcentrifuge wall during centrifugation.

The use of other manufactured tubes in the FastPrep® Instrument is not recommended and may result in sample loss or FastPrep® Instrument failure.

Add the RNA*pro*™ Solution to the sample as soon as possible to initiate RNase inhibition. Samples, both FastPrep® Instrument homogenized and non-homogenized, are stable in RNA*pro*™ Solution overnight at room temperature or 4ºC.

Bacterial strain variability may result in unwanted protein and mucopolysaccharide carryover into the aqueous solution following chloroform extraction. While this may not compromise downstream applications the user may adapt the protocol to include an additional chloroform (isoamyl alcohol may be included with the chloroform [CHCl3:IAA, 24:1, v:v]) extraction after Step 10 (Quick Protocol for Experienced Users) or in Step 12 (Detailed Procedure) to reduce the potential carryover.

A single 40 second run at a speed setting of 6.0 in the FastPrep® Instrument is sufficient to lyse a bacterial sample. If the user determines that additional processing steps in the FastPrep® Instrument are required to homogenize a sample it is recommended that the sample be incubated on ice in the sample tube for at least 2 minutes between successive FastPrep® Instrument homogenizations to prevent sample heating and possible RNA degradation.

The FastRNA® Pro Blue Kit is designed to selectively purify total cellular RNA from DNA and protein. Experiments have indicated the RNA is sufficiently pure for use in RT-PCR and northern analysis, however, it is recommended the user incorporate DNase I treatment of the RNA prior to use in applications where absolute control of DNA contamination is essential. Use DNase I at the concentration recommended by the manufacturer and incubate at 37ºC for 30 minutes. The DNase I is inactivated by incubation at 75ºC for 5 minutes or by addition of EDTA to 25 mM followed by phenol/chloroform extraction and precipitation (2, 3).

4. Safety Precautions

The RNA*pro*™ Solution contains components that when in contact with human tissue or during inhalation may cause irritation or burning. Wear personal protective equipment to prevent skin contact (e.g., gloves, lab coat, and eye protection) and prevent inhalation of reagent vapors and consumption of liquid during use. Consult the enclosed Material Safety Data Sheet for additional details.

5. Quick Protocol for Experienced Users

1. Dilute 1 ml of an overnight bacterial culture into 14 ml of fresh media in a sterile 50 ml tube and incubate for ~4–6 hours to reach an OD_{600} = 0.9–1.0.

2. Remove 10 ml of the culture to a 15 ml conical tube and pellet the cells by centrifugation. Decant the supernatant and add 1 ml of RNA*pro*™ solution to the tube and resuspend the cells by pipetting or vortexing.

3. Transfer 1 ml of the bacterial solution mixture to a blue-cap tube containing Lysing Matrix B provided in the kit.

4. Process the tube in the FastPrep® Instrument for 40 seconds at a setting of 6.0.

5. Remove and centrifuge the tube at a minimum of 12,000 x g for 5 minutes at 4ºC.

6. Transfer the liquid (~750 µl) to a new microcentrifuge tube. Avoid transferring the debris pellet and lysing matrix.

7. Incubate the transferred sample 5 minutes at room temperature.

8. Add 300 µl of chloroform (NO isoamyl alcohol). Vortex 10 seconds and then incubate 5 minutes at room temperature.

9. Centrifuge the tubes at a minimum of 12,000 x g for 5 minutes at 4ºC.

10. Transfer the upper phase (without disturbing the interphase) to a new microcentrifuge tube.

11. Add 500 µl of cold absolute ethanol, invert 5X to mix and store at -20ºC for at least 30 minutes.

12. Centrifuge at a minimum of 12,000 x g for 15 minutes at 4ºC and remove the supernatant.

13. Wash the pellet with 500 µl of cold 75% ethanol (made with DEPC-H_2O).

14. Remove the ethanol, air dry 5 minutes at room temperature (DO NOT completely dry the RNA) and resuspend the RNA in 100 µl of DEPC-H_2O.

15. Incubate 5 minutes at room temperature.

16. Determine the RNA concentration:
    a. Dilute 5 µl of RNA into 495 µl of DEPC-H_2O
    b. Read the OD_{260} using DEPC-H_2O as a blank
    c. Calculate the sample µg RNA per ml using the formula:
    $(OD_{260})(40\ \mu g/ml/per\ OD)(100\ [dilution\ factor]) = \mu g\ RNA\ per\ ml$

17. Aliquot and store the RNA solution at -70ºC.

18. RNA integrity can be analyzed visually using denaturing or non-denaturing 1.2% agarose gel electrophoresis (See Figure 1) (2, 3).

6. Detailed Protocol

1. Dilute 1 ml of an overnight bacterial culture into 14 ml of fresh media in a sterile 50 ml tube or 250 ml flask.

2. Incubate for ~4–6 hours at 37ºC with shaking at ~150–200 rpm to reach an OD_{600} = 0.9–1.0 (Note: 1.0 OD_{600} for Escherichia coli is ~1 X 10^9 cells per milliliter).

3. Remove 10 ml of the culture to a 15 ml conical tube and pellet the cells by centrifugation at 2,800 rpm (x 1,500 g) for 15 minutes at 4ºC (e.g., Beckman Model TJ-6 Centrifuge, I-92 Swinging Bucket Rotor) for 10 minutes.

4. Decant the supernatant and add 1 ml of RNA*pro*™ Solution to the tube. Completely resuspend the cells by pipetting or vortexing.

5. Transfer 1 ml of the resuspended cells to a blue-cap tube containing Lysing Matrix B provided in the kit. Securely close the cap to prevent leakage in the next step. NOTE: The calculated volumes will provide adequate airspace in the matrix tube to prevent sample leakage and/or tube failure. DO NOT overfill the matrix tube. To process a greater number of cells use a second matrix tube.

6. Process the sample tube in the FastPrep® Instrument for 40 seconds at a setting of 6.0.

7. Remove the sample tube and centrifuge at a minimum of 12,000 x g for 5 minutes at 4ºC or room temperature.

8. Transfer the liquid (~750 µl) to a new microcentrifuge tube. Avoid transferring the debris pellet and lysing matrix.

9. Incubate the transferred sample 5 minutes at room temperature to increase RNA yield.

10. Add 300 µl of chloroform (NO isoamyl alcohol). Vortex 10 seconds.

11. Incubate 5 minutes at room temperature to permit nucleoprotein dissociation and increase RNA purity.

12. Centrifuge the tubes at a minimum of 12,000 x g for 5 minutes at 4ºC. Samples containing large amounts of cellular mucopolysaccharides can be re-extracted with chloroform (isoamyl alcohol may be included with the chloroform [CHCl3:IAA, 24:1, v:v]) to increase RNA purity. Alternatively, a lithium chloride precipitation may be used (see the Troubleshooting section and references 3, 4).

13. Transfer the upper phase to a new microcentrifuge tube without disturbing the interphase. If a portion of the interphase is transferred, repeat the centrifugation with the upper phase, and transfer the new upper phase to a clean microcentrifuge tube.

14. Add 500 µl of cold absolute ethanol to the sample, invert 5X to mix and store at -20ºC for at least 30 minutes.

15. Centrifuge at a minimum of 12,000 x g for 15 minutes at 4ºC and remove the supernatant. The RNA will appear as a white pellet in the tube. If the pellet is floating the sample may be recentrifuged to place the pellet at the tube bottom.

16. Wash the pellet with 500 µl of cold 75% ethanol (made with DEPC-H_2O).

17. Remove the ethanol, air dry 5 minutes at room temperature (DO NOT completely dry the RNA) and resuspend the RNA in 100 µl of DEPC-H_2O for short-term storage. RNA is generally stable for up to a year at -80ºC. For longer term storage RNA samples may be stored at -20ºC as ethanol precipitates. When stored as an ethanol precipitate, the RNA must be precipitated and resuspended in aqueous solution prior to use. NOTE: RNA does not evenly distribute in ethanol and can lead to inconsistent RNA amounts between samples when equal volumes are pipetted. Vortex the RNA:ethanol solution to disperse the RNA prior to removing the sample. In situations where precise amounts of RNA are required it is best to precipitate the total amount (or excess) of RNA required, resuspend the RNA in DEPC-H_2O and measure the concentration by OD_{260} before proceeding.

18. Incubate 5 minutes at room temperature to facilitate RNA resuspension.

19. Determine the RNA concentration:

a. Dilute 5 µl of the purified RNA into 495 µl of DEPC-H_2O
b. Read the OD_{260} using DEPC-H_2O as a blank
c. Calculate the sample µg RNA per ml using the formula:
(OD_{260})(40 µg/ml/per OD)(100 [dilution factor]) = µg RNA per ml

Spectrophotometer accuracy is greatest between ~0.2 and ~0.8. If the OD reading is below the range, add more RNA sample (e.g., 20 µl RNA + 480 µl DEPC-H_2O) or concentrate the RNA by precipitation and resuspension into a smaller volume. If the OD reading is above the recommended spectrophotometer range, use less RNA for the OD determination.

20. Aliquot and store the RNA solution at -70ºC.

21. The RNA integrity can be determined by analyzing a portion of the RNA sample using gel electrophoresis. Add 1 µg RNA in 9 µl DEPC-H_2O, heat to 65ºC for 5 minutes, add gel loading buffer (see Related Products) and load the sample on a 1.2% agarose gel containing 2.2M formaldehyde in MOPS buffer. The sample is run at ~ 80 volts for 30 minutes (2, 3). Ethidium bromide may be added to the denatured RNA sample at a final concentration of 10 µg per milliliter prior to gel loading or the gel may be ethidium bromide stained and destained following electrophoresis and visualized under UV light. The quality of the RNA is determined by the appearance of the large, 23S, and small, 16S ribosomal RNAs as sharp, distinct bands of 2.9 and 1.5 kb. Heterogeneous-sized messenger RNA may appear as diffuse ethidium staining between and below the ribosomal bands. Small RNA species such as tRNA and 5S RNA may be present in varying amounts at the dye front.

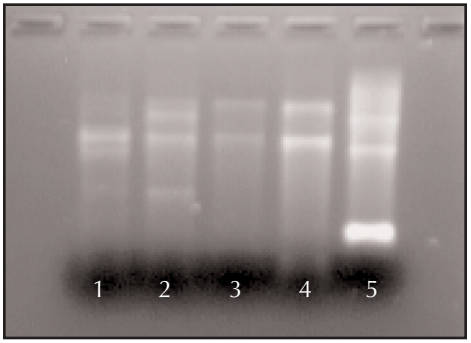

Figure 1: Bacterial total RNA extracted with the FastRNA® Pro Blue Kit. Approximately 2% of the total RNA isolated from 10^{10} bacterial cells was loaded on to a 1.2% denaturing agarose gel (1XMOPS). Lane 1: Salmonella typhimurium; Lane 2: Pseudomonas stuartii; Lane 3: Escherichia coli; Lane 4: Bacillus subtilis; Lane 5: 0.24-9.5kb RNA Ladder.

7. Troubleshooting

7.1 Degraded RNA or Lower than Expected RNA Yields

RNA purified using the FastRNA® Pro Blue Kit and analyzed by denaturing or non-denaturing agarose gel electrophoresis will appear as 2 distinct ribosomal RNA (rRNA) bands of approximately equal fluorescent intensity using ethidium bromide staining. The rRNA bands will appear in the area between 2000 and 1000 nucleotides. Messenger RNA (mRNA), which typically represents approximately less than 1% of the total cellular RNA and is heterogeneous length, will not be visible as distinct bands. rRNA is used as a marker to assess sample RNA degradation. Degraded RNA or mRNA may appear as unequal fluorescent intensity between bands, a single band may be completely lacking or a heterogeneous fluorescent smear may appear below the rRNA bands or throughout the gel lane.

Recommended precautions include cleaning all instruments and work area with RNase Erase® (Qbiogene Catalog # 2440-204) prior to use. Use disposable sterile plastic containers when possible. Glassware should be thoroughly cleaned, rinsed with DEPC-H_2O and baked at 250ºC for 4 hours to remove RNase. Sterile, plugged micropipettes are recommended (see 2, 3 for additional suggestions).

Certain bacterial strains may contain elevated RNase levels. Reduce the exposure time to RNase by adding RNA*pro*™ Solution to each sample as soon as possible following sample harvest. Process fewer samples to shorten the time before complete cellular lysis and exposure to the RNase inactivating activity of RNA*pro*™ Solution.

Bacteria in log phase growth with maximal aeration and nutrients provide the highest yield and integrity RNA. Bacterial cells in stationary phase, growing in oxygen or nutrient limiting conditions, stored for extended duration at room temperature or refrigerated for extended periods will contribute to reduced RNA yield and integrity.

RNA*pro*™ Solution can permeate samples and will protect bacterial RNA from degradation for at least 24 hours before it is processed in the FastPrep® Instrument.

Artifactual RNA degradation may occasionally occur during gel electrophoresis due to a gel that was not RNase free, running the gel at too high voltage or from using depleted running buffer. Rerun the samples with a known intact RNA sample using freshly prepared reagents.

RNA degradation may occur due to RNase contamination introduced into the DEPC-H_2O following use. If contamination is suspected, prepare fresh DEPC-H_2O in an RNase free container (2, 3). RNA*pro*™ Solution contains RNase inactivating components and will not support active RNase contamination.

7.2 No Pellet after Ethanol Precipitation

The purified RNA may not appear as a pellet but may instead adhere to the side of the tube. The RNA may not be visible and it MAY APPEAR THAT RNA HAS NOT BEEN PURIFIED. COMPLETE THE RNA PURIFICATION per the instructions provided and confirm the RNA concentration by OD_{260} and integrity by gel electrophoresis. RNA adhering to the tube wall will not affect its purity, size or use in subsequent applications.

The RNA pellet may not be firmly attached to the side of the tube and may be observed floating in the solution or at the solution surface. Recentrifuge the sample in the same tube and exercise caution to not lose the pellet when removing the supernatant.

Confirm enough sample was used to isolate RNA (1.0 OD for *Escherichia coli* ~$1x10^9$ cells).

7.3 Genomic DNA Contamination

Genomic DNA contamination will appear as a high molecular weight smear on a denaturing gel or as ethidium bromide stained material in the gel loading well. In the event genomic DNA contamination occurs, treat sample with DNase according to the manufacturer's instructions.

7.4 Mucopolysaccharide / Carbohydrate Contamination

Samples containing large amounts of cellular mucopolysaccharides can be re-extracted after the initial chloroform extraction with a second chloroform extraction. Isoamyl alcohol may be included with the chloroform [CHCl3:IAA, 24:1, v:v]) to increase RNA purity. Refer also to Lithium Chloride Precipitation in the Troubleshooting section.

7.5 Lithium Chloride Precipitation

Lithium chloride (LiCl) may be used to precipitate RNA while excluding carbohydrate, DNA and proteins, including transcription inhibitors. Lithium chloride has historically been used to precipitate RNA greater than ~300 nucleotides from tRNA and 5S RNA. Lithium chloride precipitation may be incorporated into the FastRNA® Pro Blue Kit procedure: Following ethanol precipitation of the RNA and resuspension in 100 µl

DEPC-H_2O, add lithium chloride to a final concentration of 2–3 M (e.g., 0.2 volumes [20 µl] RNase free 8 M lithium chloride). Add 2.5 volumes RNase free absolute ethanol (250 µl). Mix the solution and store on ice at least 2 hours. Centrifuge for 15 minutes at a minimum of 12,000 rpm at 4ºC. Remove the supernatant and wash the pellet with 75% cold RNase free ethanol. The ethanol wash step is critical to prevent LiCl inhibition of cell-free translation and in vitro transcription. Air dry and resuspend the RNA in 100 µl DEPC-H_2O.

8. Recommended Reference Format for Publications

Total RNA was isolated from _____ mg of _____cells using the FastRNA® Pro Blue Kit (Qbiogene, Inc., CA) and FastPrep® Instrument (Qbiogene, Inc, CA), for _____ seconds at a speed setting of _____.

9. References

1. *U.S. Patent 5,567,050. Zbloninsky et.al, Apparatus and method for rapidly oscillating specimen vessels.*
2. *Molecular Cloning, Sambrook and Russell. Cold Spring Harbor Laboratory Press, 3rd Edition, 2001.*
3. *Current Protocols in Molecular Biology, John Wiley & Sons, Inc., 2002, www.currentprotocols.com.*

10. Related Products

Description	Size	Catalog #
FastPrep® FP100A Instrument	100V	6001-100
FastPrep® FP120A Instrument	120V	6001-120
FastPrep® FP220A Instrument	220V	6001-220
FastRNA® Pro Red Kit (Yeast)	50 preps	6035-050
FastRNA® Pro Green Kit (Plant & Animal)	50 preps	6045-050
FastRNA® Pro Soil Kit	50 preps	6070-050
FastDNA® Kit	100 preps	6540-400
FastDNA® SPIN Kit for Soil	50 preps	6560-200
FastPROTEIN™ Blue Matrix	50 preps	6550-400
FastPROTEIN™ Red Matrix	50 preps	6550-600
RNase Erase®	500 ml	2440-204
Lysing Matrix B	50 x 2ml tubes	6911-050
BGFNE (alkaline agarose gel loading dye)	1 ml	2339-104
BBXFE (denaturing RNA gel loading dye)	1 ml	2343-104
BBG (general purpose neutral gel RNA and DNA loading dye)	1 ml	2327-104
BBG (general purpose neutral gel RNA and DNA loading dye)	1 ml	2327-104

11. Product Use Limitation & Warranty

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