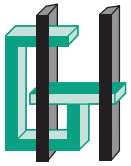
MessageClean®

For complete removal of DNA contamination from RNA
(for research use only)



GenHunter® Corporation

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The mRNA Differential Display process is covered by U.S. Patents 5,262,311; 5,599,672; 5,665,547; 5,965,409; Japanese Patent 2843675; and other issued or pending foreign patents exclusively licensed to GenHunter Corporation. Purchase of the RNAimage® and RNAspectra™ Kits from GenHunter Corporation comes with a sub-license to practice the differential display process.

The PCR process is covered by patents owned by Hoffmann-LaRoche, Inc. Use of the process requires a license.

Introduction

It is crucial that the total RNA used for mRNA Differential Display (DD) be absolutely free of DNA contamination (1, 2, 3). The same is also recommended for RNA to be used for RT-PCR or DNA Microarray.

Regardless of the method used for RNA isolation, a cleaning step is necessary to ensure the removal of this DNA contamination, especially if the differential display banding pattern on the denaturing polyacrylamide gel is seen to be independent of the reverse transcription step. GenHunter strongly recommends the use of the MessageClean® kit routinely before using the RNAimage® or RNAspectra™ kit for mRNA Differential Display (U.S. Patents 5,262,311; 5,599,672; 5,665,547; 5,965,409; Japanese Patent 2843675; and pending foreign patents).

Precaution

The DNase I sold by nearly all vendors, though claimed to be RNase free, contains detectable RNase activity. The MessageClean® kit from GenHunter Corporation contains everything needed to remove the DNA contamination from RNA samples and we guarantee that the RNA will be intact following digestion with GenHunter DNase I.

A: Other vendors' DNase I **B: GenHunter's DNase I**

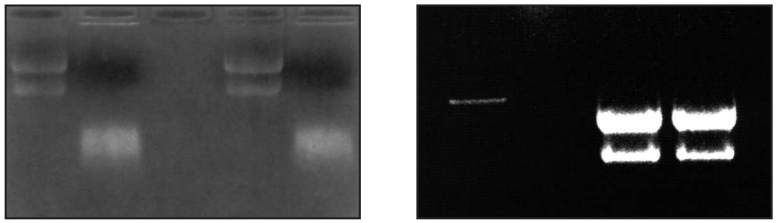

Figure 1: **Panel A** shows total cellular RNA before and after treating with 10 units of DNase I from two major vendors. Both RNA samples were degraded after treatment. **Panel B** shows the result of GenHunter's stringent QC with the MessageClean® Kit. λ-DNA (lanes 1 and 2) or total RNA (lanes 3 and 4) were incubated without (lanes 1 and 3) or with (lanes 2 and 4) 10 units GH-DNase I. As expected, only the λ-DNA, not the RNA, was degraded.

Components

(for 20 RNA sample cleanings)

1. 10X Reaction Buffer	140 µL
2. GH-DNase I (RNase free) 10 units/µL	20 µL
3. 3M NaOAc	140 µL
4. DEPC-treated H_2O	1 mL
5. RNA Loading Mix	400 µL

Instructions

I. DNase I Digestion

Add in order	µL
Total RNA*	50 (10 - 50µg)
10X Reaction Buffer	5.7
DNase I (10 units/µL)	1.0
Total	56.7

*Use DEPC-treated H_2O when diluting RNA.

Mix well and incubate for 30 minutes at 37˚C.

II. Phenol/$CHCl_3$ (3:1) Extraction

This step is essential to ensure complete removal of protein contamination and the DNase I from the RNA sample.

1. Add 40 µL of Phenol/$CHCl_3$. Vortex for 30 seconds. (See Appendix I for instructions to make Phenol/$CHCl_3$ yourself.)
2. Let sit for 10 minutes on ice.
3. Spin in a centrifuge at 4˚C for 5 minutes (maximum speed).
4. Collect and save the upper phase.

III. Ethanol Precipitation

1. Add 5 µL of 3M NaOAc and 200 µL 100% EtOH. Mix well!
2. Let sit at least 1 hour at -80˚C. Overnight to a few days at -80˚C is fine.
3. Spin for 10 minutes at 4˚C to pellet the RNA.
4. Carefully remove the supernatant and rinse the RNA pellet with 0.5 mL of 70% EtOH (in DEPC-H_2O). Do not disturb the RNA pellet!
5. Spin for 5 minutes, remove EtOH. Spin again briefly, then remove the residual liquid.
6. Re-dissolve RNA in 10 to 20 µL DEPC-H_2O.

IV. RNA Quantification and Integrity Verification

1. Quantitate by OD_{260} after 1:1000 dilution of the cleaned RNA sample.
2. Check the integrity of the RNA by running out 2-3 µg of RNA sample on a 7% formaldehyde agarose gel (see Appendix I for preparation) with the RNA Loading Mix (included in the kit):

   a) Add 1 - 10 µL (2 -3 µg) of RNA to 20 µL RNA Loading Mix. Mix well.
   b) Incubate at 65˚C for 10 minutes.
   c) Spin down samples.
   d) Put samples on ice for 5 minutes.
   e) Mix again and load entire amount onto RNA gel.

   * There is no need to add ethidium bromide to gel, the RNA Loading Mix already contains it.

   Look for the clear appearance of the 28S and 18S rRNA bands (See Figure 1B).
3. It is recommended that you store your RNA samples as 1 to 2 µg aliquots at -70˚C to prevent repeated freezing/thawing. If using for differential display, dilute the appropriate amount of RNA to 0.1 µg/µL with DEPC-H_2O just before usage. Do not re-use the diluted RNA (0.1 µg/µL) after freezing and thawing.

Appendix I: Reagent Preparations

1. Preparation of an RNA gel (7% formaldehyde, 1% agarose, 1X MOPS):

a) Add the following to a microwave-safe container:

10X MOPS Buffer	10 mL
agarose	1 g
H_2O	83 mL

b) Microwave for approximately 3 minutes or until agarose is melted.

c) Let agarose cool down to about 50˚C (barely touchable with your hand).

d) Add 7 mL of formaldehyde.

e) Gently swirl the flask to mix, then pour the gel.

f) Use 1X MOPS as the running buffer.

Note: It is not necessary to add ethidium bromide to the gel if you are using the RNA Loading Mix, which already contains it.

2. Preparation of Phenol/$CHCl_3$ (3:1), Tris-saturated, pH 7.0:

a) Melt crystalline phenol at 65˚C.
b) Add 30 mLs of the melted phenol to 10 mLs chloroform.
c) Add 10 mLs 1M Tris-HCl, pH 7.0.

3. Ordering information for additional kits and/or individual components:

Item	Amount	Cat. No.
MessageClean® Kit	1 kit	M601
10X Reaction Buffer	140 µL	R102
3M NaOAc	140 µL	R103
RNA Loading Mix	1 mL	R104
DEPC-treated H_2O	1 mL	R105

Appendix II: Trouble-Shooting Guide

GenHunter recognizes that RNA degradation may occur despite precautions taken. We are committed to providing our customers with RNase-free DNase I. If you experience degradation, please review the following possible causes and solutions first. If further assistance is needed, please contact GenHunter technical support.

Possible causes of degradation and their solutions:

1. RNA degraded before treatment with DNase I. Check integrity at all stages (before digestion, after digestion, after Phenol/$CHCl_3$ extraction, etc.). Always store RNA at -80˚C at concentrations of at least 1 µg/µL.

2. Used DNase I other than GH-DNase I. DNase I from other vendors often contains detectable RNase contamination. Use GH-DNase I.

3. RNA degraded by reagents or equipment other than reagents provided in the kit. Include a control in which the RNA is not treated with any solution from the kit, but still undergoes treatment with Phenol/$CHCl_3$ and ethanol. Make sure all solutions and buffers are made with DEPC-treated dH_2O and all vessels including tubes, tips, and gel boxes are free of RNase.

4. The RNA sample itself is contaminated with RNase. This is a common problem with RNA extracted from large amounts of tissue. To confirm, incubate RNA with 1-2 mM $MgCl_2$ in Tris-Cl pH 8.0 at 37˚C for 30 minutes. This will activate any RNase in the RNA. Solution: Lower the ratio of tissue to RNA extraction solution (RNApure®, RNAzol®, TRIzol®, TRI Reagent®, Ultraspec®, etc.) or repeat the phenol/$CHCl_3$ extraction with RNA sample.

5. The RNA sample sometimes appears to be degraded after agarose gel analysis, when the actual problem is the pH of the buffer, too much salt in the RNA, or bad loading dye, which has caused the ribosomal RNA's (28S and 18S) to migrate strangely. Solutions: Use GenHunter's RNA Loading Mix (included in kit - Reorder Cat. No. R104 for 1 mL size). Confirm the pH of the MOPS buffer (should be between 6.5 and 7.0). Make sure formaldehyde is added to the gel and the RNA sample is denatured by incubating in RNA Loading Mix at 65˚C for 10 minutes before loading.

Appendix III: Frequently Asked Questions

1. **Which RNA isolation kit is recommended?**
   GenHunter's RNApure™ Reagent (Cat. No. P501 or P502) works extremely well for RNA isolation. This is a monophasic solution based on a phenol/guanidine thiocyanate solution similar to others on the market, but less expensive.

2. **What is the pH of the NaOAc contained in the kit?**
   The pH is 5.5.

3. **What is the composition of 10X Reaction buffer?**
   100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin.

4. **Can Phenol/$CHCl_3$ (1:1) or Phenol/$CHCl_3$/isoamyl alcohol (25:24:1) be used in place of Phenol/$CHCl_3$ (3:1)?**
   Yes, but in this case the extraction should be repeated twice to insure complete removal of proteins. Phenol/$CHCl_3$/isoamyl alcohol is normally used for DNA or plasmid purification. It is recommended that all reagents for RNA work be separated from DNA work to avoid RNase contamination.

5. **Where can Phenol/$CHCl_3$ (3:1) be purchased?**
   These reagents can be purchased from any supplier of high quality reagents and mixed to 3:1. See Appendix I for instructions to make Phenol/$CHCl_3$ yourself. Tris-saturated phenol is preferred (pH ~7.0).

6. **Can a heat inactivation of the DNase be done instead of the Phenol/$CHCl_3$ step?**
   No, we do not recommend this especially if using for DD or RT-PCR. The inactivation of the DNase is often incomplete and given the small amount of cDNA that is made during the RT step, any residual DNase could have a disastrous effect.

6. **How critical is the timing at each step?**
   Times may be extended, for convenience, but not shortened.

7. **What is the best way to check the integrity of the RNA?**
   Run out a few microliters of RNA sample (2-3 µg) on a 7% formaldehyde agarose gel (see Appendix I for instructions). Look for the clear appearance of 28S and 18S rRNA bands as seen in Figure 1B. If both ribosomal RNA bands disappear or become smeared, the RNA sample may be degraded.

8. **Can I use RNA samples dissolved in formamide for the cleaning?**
   No. The formamide will disrupt the enzyme activity.

9. **Is it important to check the purity of the RNA with the ratio of OD_{260}:OD_{280}?**
   When using the standard phenol/$CHCl_3$ RNA isolation kits (such as RNApure™, RNAzol®, TRIzol®, etc.), we do not find that this is very crucial. However, the ratio should be at least 1.5.

References

1. Liang, Peng et al. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Research (1993) 21: 3269-3275.

2. Liang, Peng et al. Method of Differential Display. Methods in Molecular Genetics. (1994) 5: 3-16.

3. Liang, Peng and Arthur Pardee. Recent advances in differential display. Current Opinion in Immunology (1995) 7: 274-280.

Additional references can be found on GenHunter's website at www.GenHunter.com