Efficacy of RNase inhibitors and two nucleic acid preservatives to preserve RNA in colon cancer tissue sections for laser capture microdissection

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Abstract

Laser capture microdissection is a well-established technique for procuring pure populations of cells from clinical tissue samples for gene expression analysis. Since RNA is very labile, a frequent question by investigators is the maximum amount of time they have to complete microdissection after tissue preparation and still obtain RNA of suitable quality for gene expression analysis. This study compares recovery and quality of RNA after different time intervals from untreated stained tissue sections and stained tissue sections that have been treated with RNase inhibitors and two nucleic acid preservatives.

Anonymized snap-frozen colon cancer tissue was used for this study. Eight micron tissue sections were cut onto charged glass slides. Tissue sections were hematoxylin and eosin stained as follows: no treatment; staining in the presence of one of three different commercially-available RNase inhibitors including including ProtectRNA™ (Sigma Aldrich, St. Louis, Missouri, USA), Protector RNase Inhibitor (Roche, Indianapolis, Indiana, USA), or RNase Inhibitor (Qiagen, Valencia, California, USA); treatment with one of two different nucleic acid preservatives including a novel reagent, RNAStable™(Biomatrica, Inc., San Diego, CA) or a commercially-available reagent, RNAlater® (Applied Biosystems/Ambion, Austin, TX) after staining. After staining and treatment, slides were set at room temperature for 0, 2, 24, and 48 hours for the RNase inhibitor treatments and for 0, 48 hours, 7 and 28 days for nucleic acid preservatives. After the times, laser capture microdissection was used to microdissect fifteen thousand cancer cells from each slide. RNA was extracted, quantitated, and quality determined by RNA integrity number, and reverse transcription quantitative polymerase chain reaction for actin 3':M ratio.

Microdissection proceeded without any problems for all samples. For RNase-treated samples, there was no difference in the quantity of RNA between treated and untreated samples and over time. For nucleic acid preservative-treated samples, there was a significant decrease in RNA quantity from t=0 to t=48 hrs, with no change after 7 days. After 28 days, the treated slides both showed a drop while untreated remained unchanged. For RNase-treated slides, there was no difference in RNA quality between untreated and treated slides and both showed good to excellent quality RNA up to 15 hours post staining and poor quality after 48 hours. RNAStable™-treated slides retained excellent quality RNA after 7 days and good quality after 28 days. RNAlater®-treated slides retained excellent quality RNA up to 48 hours, good quality after 7 days and very poor quality after 28 days.

For microdissected frozen colon cancer tissue, this study shows that RNase inhibitors did not improve RNA preservation in tissue, one has up to 15 hours after staining tissue sections, up to 7 days after treatment with RNAlater®, and up to 28 days after treatment for the RNAStable™ to obtain RNA useful for gene expression analysis.

Background

- Laser capture microdissection (LCM) is a well-established technique for procurement of pure populations of cells from tissue sections for downstream gene expression analysis.
- Since RNA is very labile, what is the maximum time after H&E staining before performing LCM to obtain RNA of suitable quality for gene expression analysis?
  - This would be very useful for planning experiments, especially if slides can be stained in batches
  - Does treatment of tissue sections with RNase inhibitors or nucleic acid preservatives improve preservation of RNA over time compared to untreated sections?
- Bioanalyzer: RNA integrity number (RIN) is measurement of RNA quality examining rRNA.
  - Good to excellent quality RNA from microdissected tissue required for gene expression analysis
  - RIN >/= 7-10 excellent quality RNA
  - RIN >/= 5 fair to good quality RNA
  - RIN < 5 poor quality RNA
- Quantitative RT-PCR is a direct measure of RNA quality by directly examining mRNA (reference)
  - Ratio of transcript quantity for beta actin 3'/M
  - 3' primers located at the 3' end of beta actin RNA
  - M primers located approximately 600 bp upstream of the 3' end
  - Target sequences for probes used for Affymetrix U133 Plus 2.0 arrays Human Genechips fall within 600 bp of the 3' end of genes
  - Ratio of 3'/M </= 2 excellent to quality RNA
  - Ratio of 3'/M=2- 3.5 very good to good quality RNA
  - Ratio of 3'/M=3.5-4 fair quality RNA
  - Ratio of 3'/M>4 poor quality RNA

Materials and Methods

- Eight micron thick frozen colon cancer tissue sections were cut onto charged slides
- All slides H&E stained:
  - 70% ethanol-20 sec, H2O-10 sec, Mayer's hematoxylin- 15 sec, H2O-10 sec, Bluing solution- 10 sec, 70% ethanol-10 sec, eosin Y- 5 sec, 95% ethanol twice-10 sec each, 100% ethanol twice 10 sec each, xylene- 20 sec
- For RNase-treated slides, RNase inhibitors added to the staining reagents
- For nucleic acid preservative-treated slides,nucleic acid preservative added after staining
  - RNAStable™ (Biomatrica, San Diego, CA)
    - -Applied 60 µl of RNAStable™
    - -Apply coverslip to force out bubbles placed in
    - -Centrifuge at 1500 RPM for 2 mins
  - RNALater® (Ambion, Austin, TX)
    - 80 µl RNALater® is applied to the slides; coverslipped and bubbles were removed.
    - -Sealed coverslip onto slide using nail polish.
- For RNase inhibitor-treated slides, slides set at room temperature for times= 0, 2, 24 and 48 hrs
- For nucleic acid preserrvative-treated slides, slides set at room temperature for times= 0 hrs, 48 hrs, 7 days, 28 days
- After time periods for the nucleic acid preservative-treated slides, wash in water, increasing percent ethanol, xylene
- All slides were laser capture microdissected (LCM) (Arcturus Bioengineering, Molecular Devices, Sunnyvale CA) with 3000 shots (approximately 15,000 cancer cells/dissection)
- RNA purification with RNA PicoPure Kit (Molecular Devices, Sunnyvale, CA)
- RNA quantification- Ribogreen fluorescence (Molecular probes, Eugene, OR)
- RNA quality
  - Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA)
    - RNA Integrity Number (RIN)
  - Quantitative RT-PCR beta actin using an ABI PRISM 7900HT instrument (Applied Biosystems, University Park, IL)
    - Beta actin 3'/M ratio
    - Signal measured using Sybergreen
    - Primers:
    - 3' primer set:
      - Beta actin 3' left: **5'-tcctctcccaagtccacaca-3'**
      - Beta actin 3' right: **5'-gcacgaaggctcatcattca-3'**
    - M primer set:
      - Beta actin M left: **5'-gatcattgctcctcctgagc-3'**
      - Beta actin M right: **5'-agtccgcctagaagcatttg-3'**

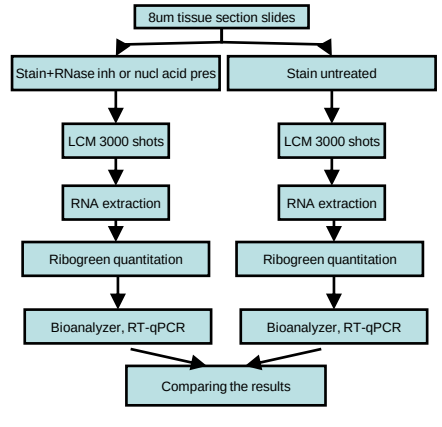

Figure 1. Schematic of protocol for efficacy of RNA preservation in tissue comparing untreated slides and slides treated with RNase inhibitors or nucleic acid preservatives.

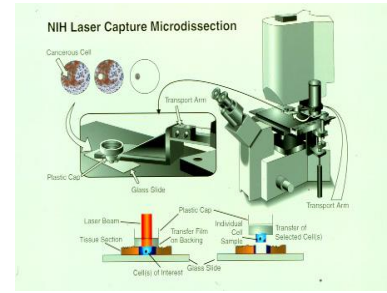

Figure 2. Schematic of laser capture microdissection apparatus and operation.

Result

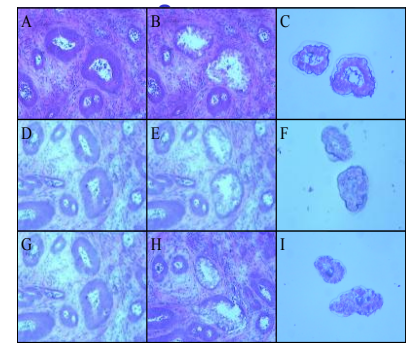

Figure 3. Laser capture microdissection of colon cancer cells. A-C Untreated slide: A.)before dissection, B.) after dissection, C.) cap with dissected cancer cells. D-F RNAStable™-treated slides: D.)before dissection, E.) after dissection, F.) cap with dissected cancer cells. G-I RNALater®-treated slides: G.)before dissection, H.) after dissection, I.) cap with dissected cancer cells.

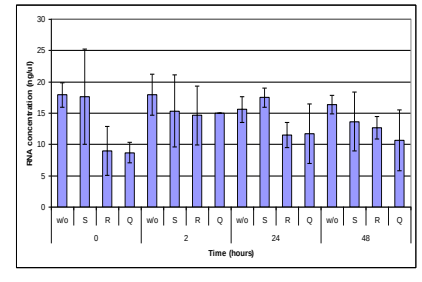

Figure 4. RNA yield for microdissected colon cancer cells over time in presence and absence of RNase inhibitors (w/o= no inhibitor, Q= Qiagen, R= Roche, S= Sigma; total volume= 20 µl per sample; error bars represent SEM; n=3 for all experiments)

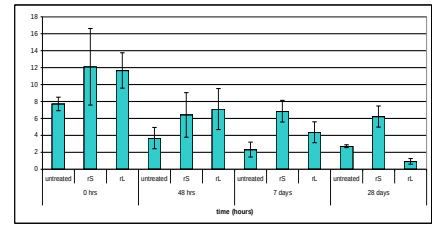

Figure 5. RNA yield for microdissected colon cancer cells over time in presence and absence of nucleic acid preservatives (untd=untreated, rS= RNAStable™, rL= RNAlater®; total volume= 20 µl per sample; error bars represent SEM; n=3 for all experiments)

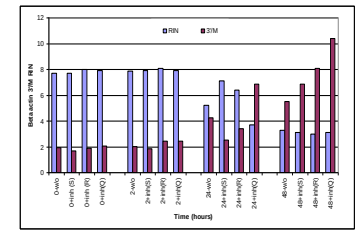

Figure 6. RNA quality as measured by RNA integrity number (RIN) and qRT-PCR beta actin 3'/M over time in presence and absence of RNase inhibitors (w/o= no inhibitor; S= Sigma; R= Roche;Q= Qiagen).

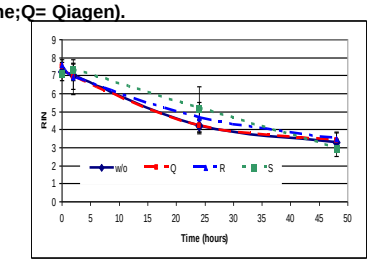

Figure 7. RNA quality as measured by RNA integrity number (RIN) over time in presence and absence of RNase inhibitors (w/o= no inhibitor; Q= Qiagen; R= Roche; S= Sigma; error bars represent SEM; n=3 for all experiments)

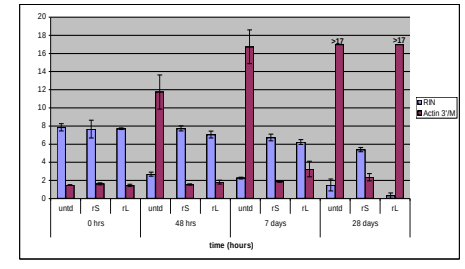

Figure 8. RNA quality as measured by RNA integrity number (RIN) and qRT-PCR beta actin 3'/M over time in presence and absence of nucleic acid preservatives (untd=untreated, rS=RNAStable™, rL=RNAlater®) Error bars represent SEM (n=3 for all experiments).

Summary

- **Tissue treatment with nucleic acid preservatives or RNase inhibitors did not affect microdissection**
- **For RNase-treated slides, no significant change in RNA yield over 48 hours between treated and untreated slides**
- **For nucleic acid preservative-treated slides, untreated and treated slides showed a decrease in RNA quantity from t=0 to t=48 hrs and no change after 7 days. After 28 days, the treated slides both showed a drop while untreated remained unchanged.**
- **For nucleic acid preservative-treated slides, RNAStable™ retained excellent quality RNA up to 7 days and very good quality after 28 days. RNAlater® retained excellent quality RNA up to 48 hours and good quality after 7 days. The untreated slides showed poor quality after 48 hours.**
- **For RNase inhibitor-treated slides, there was no significant improvement of RNA quality between different inhibitors and untreated slides and good quality RNA can be obtained up to 15 hours post staining.**

Future Directions

- **For RNAStable™- treated slide, will compare gene expression profiles between untreated T=0 and after time points**
- **Examine efficacy of RNAstable™ to preserve proteins in tissue**
- **Will examine the potential for preserving RNA in bulk tissue.**

Reference

Kube DM, Savci-Heijink CD, et al. Optimization of laser capture microdissection and RNA amplification for for gene expression profiling of prostate cancer. BMC Mol Biol. 2007 Mar 21;8 (1):25-38.