Efficacy of RNase Inhibitors to Preserve RNA in Colon Cancer Tissue Sections for Laser Capture Microdissection

Helen Zhou, BS[1], Lena Diaw, PhD[2], Hien Dang, BS[3], Helen Moore PhD[1], Jim Robb, MD, FCAP[1], Jim Vaught, PhD[1], Carolyn Compton, MD, PhD[1] John Gillespie, MD[2] 1. Office of Biorepositories and Biospecimen Research, National Cancer Institute, Bethesda, Md.2. SAIC-Frederick, Inc., Frederick, Md, 3. Penn State University, Hershey, PA.

Context: 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. Since RNA degradation is primarily due to endogenous RNases that are activated in aqueous environments, the staining process potentially leads to the greatest loss of RNA. This study will compare the efficacy of three commercially available RNase inhibitors added to hematoxylin and eosin staining reagents to preserve RNA in tissue for laser capture microdissection.

Design: Anonymized snap-frozen colon cancer tissue was used for this study. All tissue sections for microdissection were cut onto glass slides that were autoclaved and treated with RNase inhibitor. Tissue sections were hematoxylin and eosin stained without RNase inhibitor and with inhibitor using one of three different inhibitors including ProtectRNA (Sigma Aldrich, St. Louis, Missouri, USA), Protector RNase (Roche, Indianapolis, Indiana, USA), or RNase Inhibitor (Qiagen, Valencia, California, USA). Fifteen thousand cells were microdissected after being set at room temperature for 0, 2, 24, and 48 hours. RNA was extracted, quantitated, and quality determined by 28S:18S ratio, RNA integrity number, and quantitative reverse transcription polymerase chain reaction for the ratio of Cts for 3' and M primer sets.

Results: There was essentially no difference in the quantity and quality of RNA recovered from samples with and without RNase inhibitor treatment for all time points. RNA quality was excellent for 0 and 2 hours with and without RNase inhibitor treatment. After 24 hours, there was no statistical difference in RNA quality for RNase inhibitor-treated and untreated samples. After 48 hours, all samples showed poor quality RNA.

Conclusions: This study suggests that one has up to 15 hours to microdissect stained colon cancer tissue derived from frozen tissue with or without RNase inhibitor treatment to obtain RNA of suitable quality 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 molecular 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 improve preservation of RNA over time?
- Bioanalyzer: RNA integrity number (RIN) and 28S/18S ratios are measures of RNA quality examining rRNA.
  - RIN >/= 7-10 excellent quality
  - RIN >/= 5 fair to good quality
  - RIN < 5 poor quality
  - 28S/18S >/= 0.8-2.0 good to excellent quality
  - 28S/18S 0<0.8 fair to poor quality RNA
- Quantitative RT-PCR is a direct measure of RNA quality by directly examining mRNA
  - 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 very good to excellent quality RNA (reference)

Materials and Methods

- Glass slides were autoclaved and treated with RNase inhibitor (Sigma Aldrich)
- Eight micron thick frozen colon cancer tissue sections were cut onto slides
- Slides H&E stained in the presence and absence of RNase inhibitors
  - Inhibitors: ProtectRNA (Sigma-Aldrich, St. Louis, MO); RNA Protector (Roche, Indianapolis, IN), RNase Inhibitor (Qiagen, Valencia, CA)
  - Concentrations of RNase inhibitors in solutions according to manufacturer's instructions
  - Staining- sequentially pipetted 200 ul of the reagents onto the slides: 70% ethanol, H_2O, Mayer's hematoxylin, H_2O, Bluing soln, 70% ethanol, eosin Y, 95% ethanol, 100% ethanol, xylene
- Stained slides set at room temperature for times= 0, 2, 24,48 hours
- Laser capture microdissected (LCM) (Arcturus Bioengineering, Molecular Devices, Sunnyvale CA) approximately 15,000 cancer cells/dissection (3000 shots)
- RNA purification with RNA PicoPure Kit (Molecular Devices, Sunnyvale, CA)
- RNA quantitation- Ribogreen fluorescence (Molecular probes, Eugene, OR)
- RNA quality
  - Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA)
    - RNA Integrity Number (RIN)
    - 28S/18S Ratio
  - Quantitative RT-PCR beta actin using an ABI PRISM 7900HT instrument (Applied Biosystems, University Park, IL)
    - Beta actin 3'/M ratio
    - SYBER green quantitation
    - Primers:
      - Beta actin 3' left: 5'-tcctctcccaagtccacaca-3'
      - Beta actin 3' right: 5'-gcacgaaggctcatcattca-3'
      - Beta actin M left: 5'-gatcattgctcctcctgagc-3'
      - Beta actin M right: 5'-agtccgcctagaagcatttg-3'

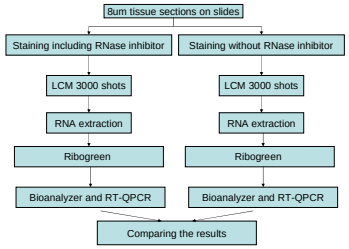

Figure 1. Schematic of protocol for efficacy of RNA preservation in tissue using RNase inhibitors

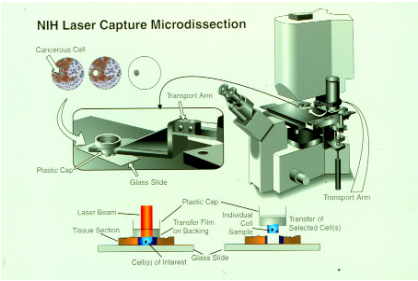

Figure 2. Schematic of Laser capture microdissection apparatus and operation.

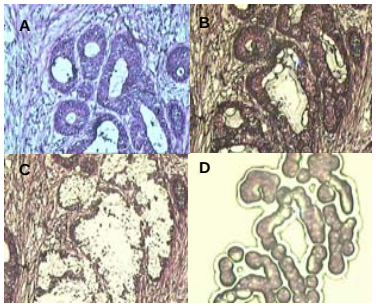

Figure 3. Laser capture microdissection of colon cancer cells. A.) Tissue before dissection, B.) after firing laser, C.) tissue area after lifting cap, and D.) cap showing adherent cells.

Results

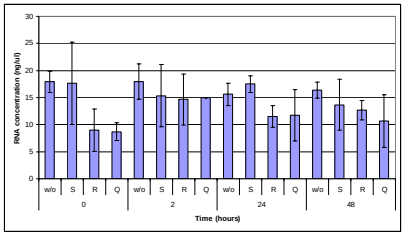

Figure 4. RNA yield for microdissected colon cancer cells over time in presence and absence of inhibitors (w/o= no inhibitor, Q= Qiagen, R= Roche, S= Sigma; total volume= 20 µl per sample; error bars represent SEM; n=3 for all experiments)

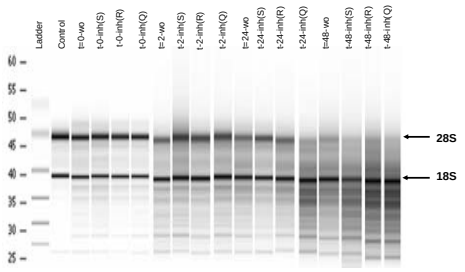

Figure 5. Electropherograms of RNA derived from microdissected cells after time and in the presence and absence of RNase inhibitors. Numbers 0, 2, 24, 48 depict time in hours and wo= no inhibitor; S= Sigma; R= Roche; Q= Qiagen. Arrows depict 28S/18S rRNA bands.

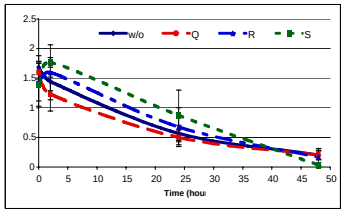

Figure 6. RNA quality as measured by 28S/18S ratios over time in presence and absence of inhibitors (w/o= no inhibitor; Q= Qiagen; R= Roche; S= Sigma). Error bars represent SEM (n=3 for all experiments).

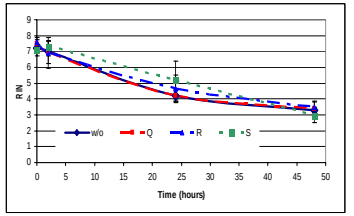

Figure 7. RNA quality as measured by RNA integrity number (RIN) over time in presence and absence of inhibitors (w/o= no inhibitor; Q= Qiagen; R= Roche; S= Sigma). Error bars represent SEM (n=3 for all experiments).

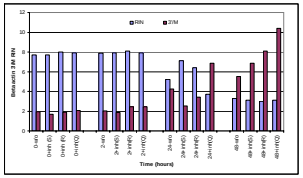

Figure 8. RNA quality as measured by RNA integrity number (RIN) and qRT-PCR beta actin 3'/M over time in presence and absence of inhibitors (w/o= no inhibitor; S= Sigma; R= Roche;Q= Qiagen).

Conclusions

- There is no relationship between quantity of recovered RNA and presence of RNase inhibitors or time.
- RNA was well preserved in H&E stained frozen colon cancer tissue sections for up to approximately 15 hours.
- Treatment of tissue sections with RNase inhibitors did not significantly improve RNA preservation in tissue.

Future Directions

- Will test other reagents for efficacy of RNA preservation in tissue
- Will examine other tissue types including tissues that contain high levels of RNases (e.g. pancreas).

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.