ANTIGENIC PROTEIN FROM RNA*LATER*® TREATED CELL CULTURES GROWN ON THE INTERNATIONAL SPACE STATION (ISS), USING THE CELLULAR BIOTECHNOLOGY OPERATIONS SUPPORT SYSTEM (CBOSS)

J.E. Love[1], D.K. Hammond[2], T.F. Elliott[2], K. Holubec[2], T.L. Baker[2], P.L. Allen[3] and T.G. Hammond[3]

[1]Biological Systems Office, NASA Johnson Space Center, Houston, TX; [2]Wyle Life Sciences, Houston, TX; [3]V.A. Medical Center, New Orleans, LA and Tulane University Health Sciences Center, New Orleans, LA.

ABSTRACT

Early CBOSS experiments on the ISS had no means provided to freeze cell culture samples. Consequently, the samples were treated with RNA*later*® (Ambion) in order to preserve RNA integrity. This study was aimed at demonstrating that protein could be recovered after removal of RNA, from cells stored long term in RNA*later*. Initial tests showed protein recovery from cell cultures stored over 3 months in refrigerated RNA*later* in amounts equivalent to that from freshly frozen and processed cultures. Matched flight (ISS Expedition 3) and ground control cell samples from Human Renal Cortical Epithelial (HRCE) cell cultures were compared. After storage in RNA*later* and extraction of RNA (RNAqueous® , Ambion), the RNA filtrate containing the protein was precipitated, washed and suspended in buffer containing SDS. Equal concentrations of protein were loaded for each sample and separated by SDS-PAGE and transferred by Western blot to PVDF membrane. The Western blots were stained with enhanced chemiluminescent ECL® Plus detection kit (Amersham) and scanned using a Storm 840 gel image analyzer (Amersham Molecular Dynamics). ImageQuant® software was used to quantify the densities of the protein bands. The ground and flight HRCE cell samples had equivalent amounts of protein, which showed a similar staining pattern over time with antibodies to the Vitamin D receptor and protein kinase C (PKC) beta isoform. (Supported by NASA Biotechnology Contract NAS9-97114 and NRA grant #NAG8-1362).

INTRODUCTION

In the early CBOSS experiments on the ISS, cell culture samples were refrigerated for storage. Consequently, the samples were fixed for later use by either formalin or RNA*later*® (Ambion). The formalin fixed cells were used to do histology and antibody staining, while the RNA*later* was used in order to preserve RNA integrity. Unfortunately the RNA that was recovered from the cells was degraded after this amount of time. This study was therefore undertaken to determine whether protein could be recovered after removal of RNA, from cells stored long term in RNA*later*. Initial tests demonstrated that protein could be recovered from cell cultures stored over 3 months in refrigerated RNA*later* in amounts equivalent to that from freshly frozen and processed cultures. Furthermore, the protein recovered was antigenic to several antibodies. Since our studies began, another group has reported use of TRIZOL to simultaneously prepare RNA and protein (1) from tissue samples stored in RNA*later*.

Matched flight (ISS Expedition 3) and ground control cell samples from Human Renal Cortical Epithelial (HRCE) cell cultures were then compared. After storage of the cells in RNA*later* and extraction of RNA (RNAqueous® , Ambion), the RNA filtrate containing the protein was precipitated, washed and suspended in buffer containing SDS. Equal concentrations of protein

were loaded on gels for each sample and separated by SDS-PAGE and transferred by Western blot to PVDF membrane. The Western blots were stained with enhanced chemiluminescent ECL Plus® detection kit (Amersham) and scanned using a Storm 840® gel image analyzer (Amersham Molecular Dynamics). ImageQuant® software was used to quantify the densities of the protein bands. Statistical analysis was done by means of an Excel graphing program using standard deviation.

When the ground and flight HRCE samples were loaded on gels with equivalent amounts of protein they showed a similar staining pattern over time with antibodies to the Vitamin D receptor (VDR), protein kinase C beta (PKCβII) isoform and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

METHODS

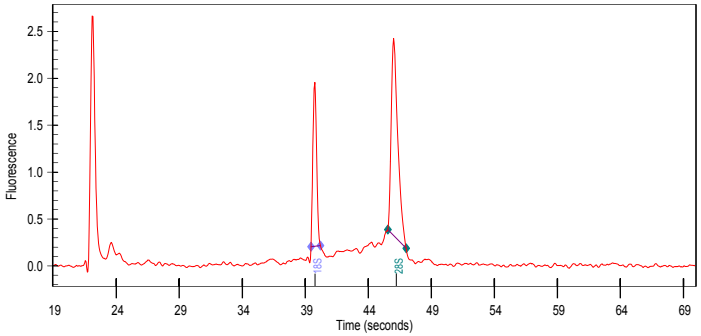
- Materials and equipment used: RNA*later*® (Ambion); RNAqueous® kit (Ambion); PBS (Dulbecco's phosphate buffered saline, w/o Ca & Mg, Celox); 96 well UV plates (Costar); SpectraMax 250 (Molecular Devices), Amersham Storm 840®, and RNase free micropipette tips, pipettes, and microcentrifuge tubes.
- Human renal cortical epithelia (HRCE) cells (2) were grown in T-flasks (Corning) or Teflon bags, TCMs (American Fluoroseal, #2P-0025) with Cytodex-3 beads (Sigma).
  - Both flight and ground cells were grown in 15mL media in TCMs. For RNA*later* treatment, as much media as possible (~10 mL) was removed and 9 mL of RNA*later* was injected into the bag which was stored in a refrigerator at 4°C for the specified time.
- For RNA preparation, a one to five milliliter aliquot of RNA*later* containing cells was placed in a centrifuge tube and diluted with an equal volume of PBS (Dulbecco's phosphate buffered saline, w/o Ca & Mg, Celox). The sample was then centrifuged and washed at 3000g for 5-10 minutes, refrigerated. The cells were immediately lysed with buffer from the RNAqueous kit (Ambion), and processed for RNA following the kit instructions.
- After removal of RNA, the filtrate (RNA-) was frozen in aliquots at –80°C until use.
- The thawed RNA- sample was diluted in half with water and precipitated with an equal volume of 10% trichloroacetic acid (TCA), washed with 5% TCA, and suspended in SDS running buffer.
- Cells that were used directly after harvesting or after being stored in RNA*later*™ were washed with PBS and suspended in SDS running buffer for initial lysis and sampling for protein determination. All samples were then treated with blue reducing buffer and boiled at 100°C for 5 minutes.

- Protein assays were run using bicinchoninic acid (BCA) (Sigma #B-9643) (3) and 4% cupric sulfate (Sigma #C-8027), 50:1, with bovine serum albumin standards (BSA) from Pierce (#23209) in a 96 well format including the detergent buffer in the standards.
- Standard Laemmli (4) sodium dodecyl sulfate polyacrylamide (SDS-PAGE) 4-20% gradient gels and 10X Tris/Glycine/SDS Buffer were purchased from BioRad. The same buffer was diluted in 20% v/v methanol-water as the transfer buffer for Western blots. A standard mini BioRad gel system was used for the SDS-PAGE and Western blots. Unless otherwise noted the gels were run at 200V and transferred to PVDF membrane (BioRad) for at least 200Vhr by the method of Towbin (5). Prestained and/or biotin labeled Bio-Rad molecular weight markers were used. When stained for protein, SYPRO Orange (Molecular Probes) was used according to manufacturer's instructions. Blots were blocked with 5% milk (6) in 0.1% Tween 20 (BioRad)-PBS(T-PBS)(5), and washed in T-PBS. Antibodies used for the Westerns were rabbit anti-vitamin D receptor rabbit and anti-Protein Kinase CβII (Santa Cruz Biotech), anti-tyrosine hydroxylase (Chemicon) and rabbit anti-GAPDH (abcam). Second antibodies were all horseradish peroxidase (HRP) conjugates specific for the goat anti-rabbit IgG (Sigma) used at 1:1000 dilution in PBS-Tween buffer. Gels stained with SYPRO Orange (Molecular Probes) and Western blots, developed with ECL™ Plus kit (Amersham), were recorded using a Storm 840 scanner (Amersham).
- The gels were quantitated using ImageQuant software. The pixel volumes minus background were used to calculate the percent of the TCM control, which was used for comparison of gels due to the inherent variability between blots. The graphs with standard deviation were prepared in Excel.

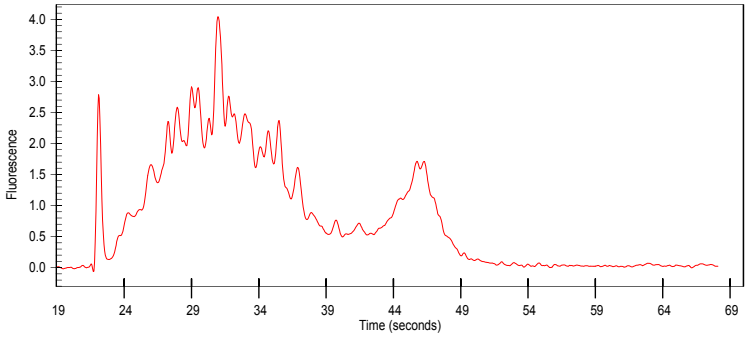
RESULTS AND FIGURES

Figure 1 - RNA prepared from 7A.1 Ground and Flight samples.
Cells were preserved in RNA*later* for 3 months at refrigerated temperatures before being frozen at –80ºC. RNA was prepared using an Ambion RNAqueous kit. This histogram shows 28S and 18S peaks detected by Agilent Bioanalyzer ® 2100 software. The control demonstrates good 18S and 28S ribosomal peaks from freshly prepared HRCE RNA. Both the ground and flight samples were degraded as shown by the day 6 examples below.

Control



Ground d6



Flight d6

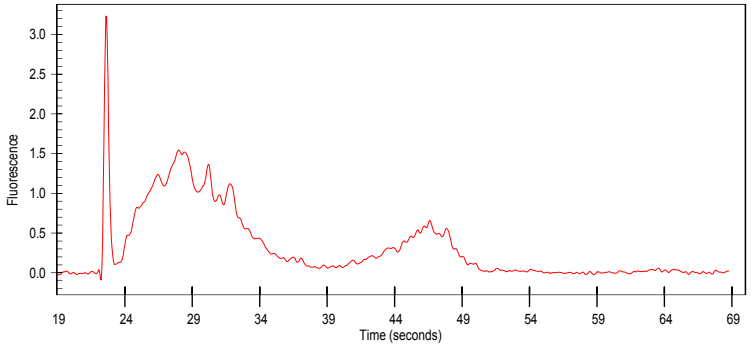


Figure 2 - Protein prep from RNA*later* treated cells

Whole cells treated with RNA*later* were washed and lysed with SDS loading buffer and run on SDS/PAGE:

A. Cellular protein (lane 4) next to molecular weight standards (lane 5) demonstrate that protein could be recovered from cells treated in this way.

B. HRCE cellular protein transferred to nitrocellulose and stained with an antibody to PKCβ, demonstrate that the protein recovered was antigenic.

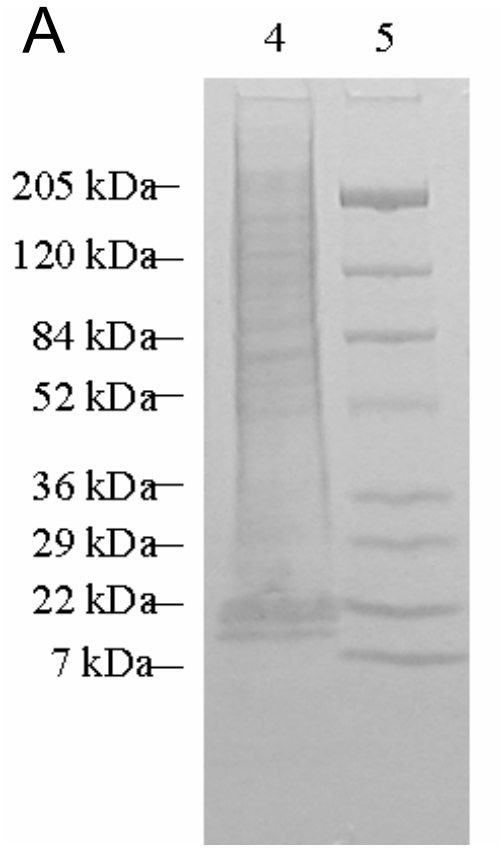


Figure 3 - Protein preparation after removal of RNA (RNA-)
Samples from ground (G) and flight (F) HRCE cell runs that had RNA removed were TCA precipitated and suspended in SDS running buffer. Molecular weight standards were run in lane 1 and 10 micrograms of protein were placed in all other lanes below. The SDS/PAGE gel was stained using SYPRO Orange and recorded on an Amersham Storm 840, demonstrating that protein could be recovered from lysates after RNA removal.

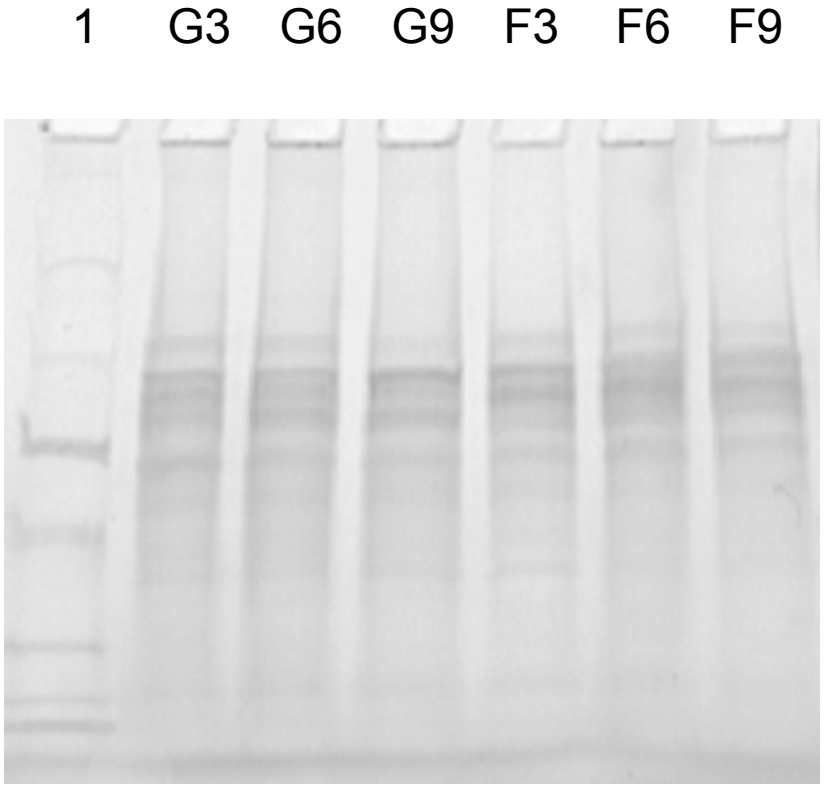
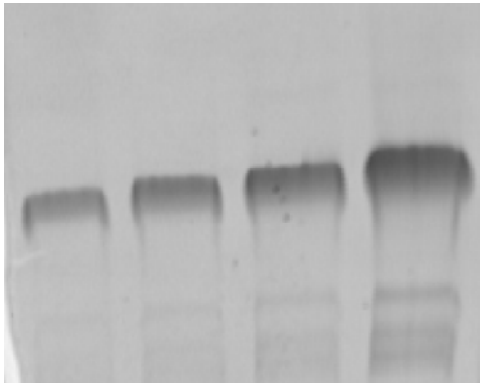


Figure 4 - Increasing Bovine Serum Albumin (BSA) concentrations. Increasing amounts of BSA were used to determine whether volume measurements of the bands on a gel resulted in adequate quantitative data. Panel A is the SYPRO Orange stained gel containing increasing amounts of BSA. Panel B shows the linear relationship between the pixel volume data, collected from equal sized rectangles, and the concentration of BSA loaded.

A



B

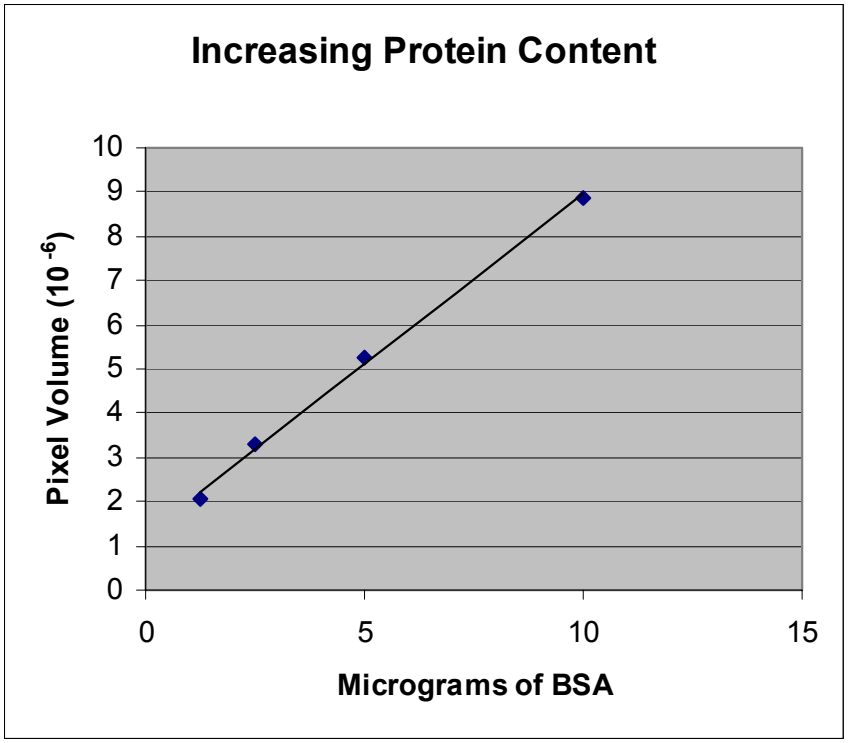


Figure 5 - Ground and Flight proteins stained with SYPRO Orange
Equal amounts of protein from ground and flight samples, prepared by TCA precipitation of RNA- samples were loaded on an SDS/PAGE gel, run and stained with fluorescent dye. The lane labeled TCM contained untreated control cells. This graph is the average of two protein stained gels. The protein loads of the flight and ground samples are roughly equivalent. The control lane (TCM) usually appears to be slightly higher in protein content.

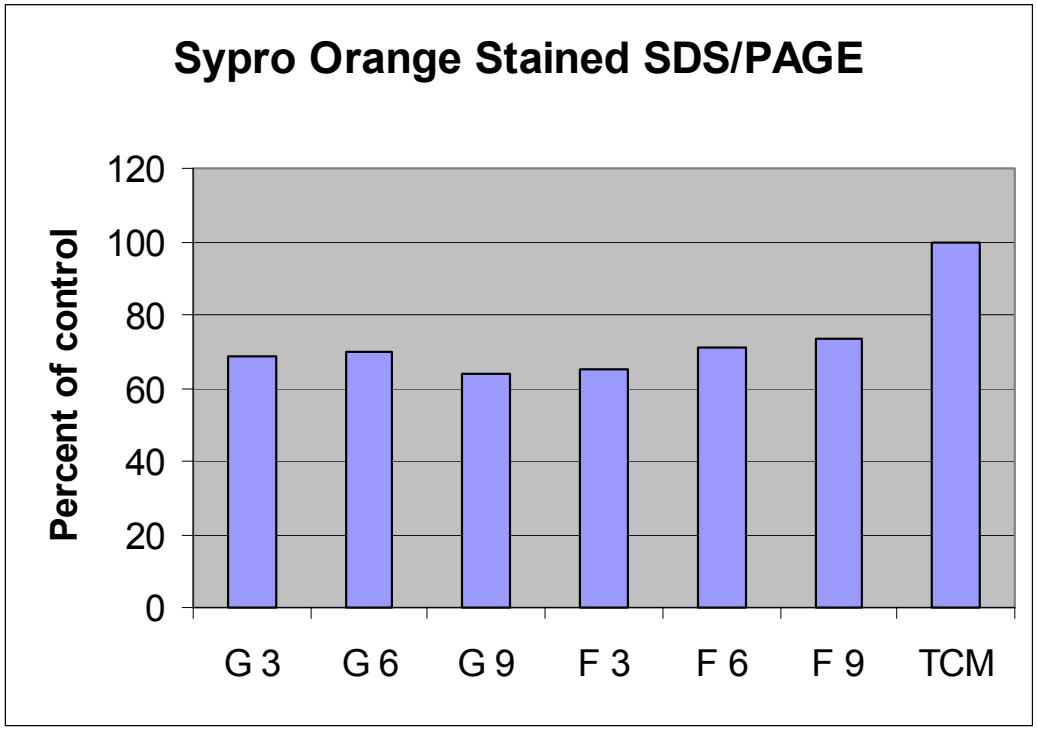


Figure 6 - Vitamin D Receptor (VDR) antibody stained blots
SDS/PAGE gels prepared with equal loading of protein were blotted and stained with anti-VDR as the first antibody. The graph below demonstrates the average of three blots. An example of one of the blots is shown below the graph, where 5 µg/lane was loaded and the VDR gave a molecular weight of 60-64kD. Except for the ground day 3 experiment, all other samples were in the same range of vitamin D Receptor per protein loaded.

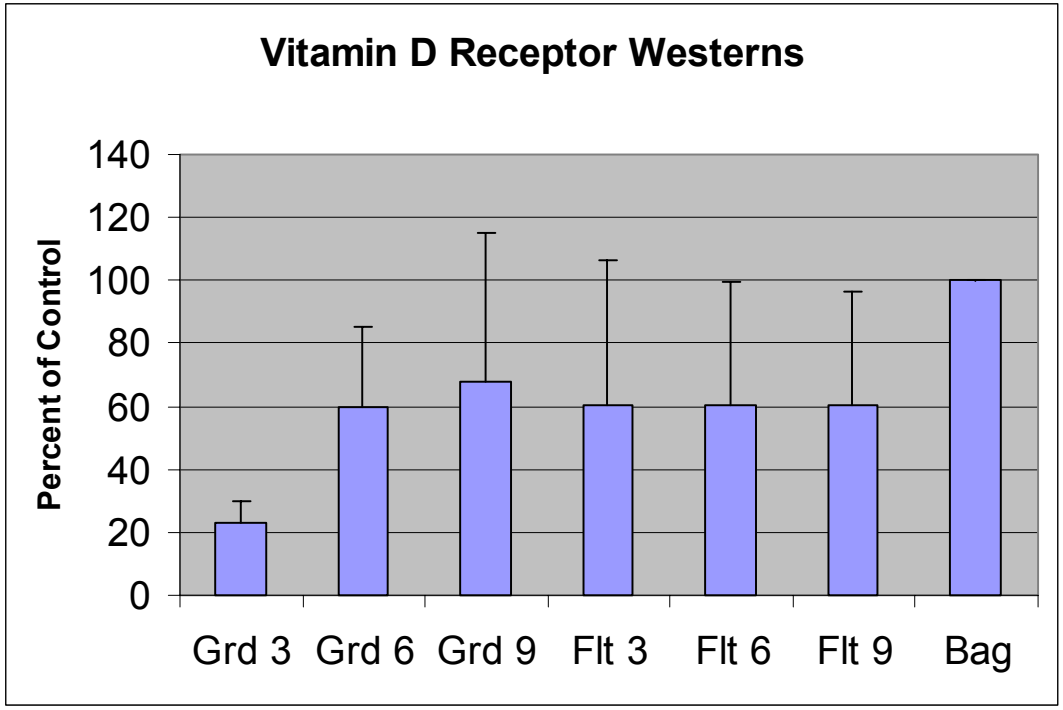


Figure 7 - Protein Kinase CβII (PKCβII) antibody stained blot. SDS/PAGE gels prepared with equal loading of protein were blotted and stained with anti-PKCβII as the first antibody. The graph demonstrates the average of four blots. An example of one of the blots is shown below the graph, where 6 µg/lane was loaded and the PKCβII gave a molecular weight of 80 kD.

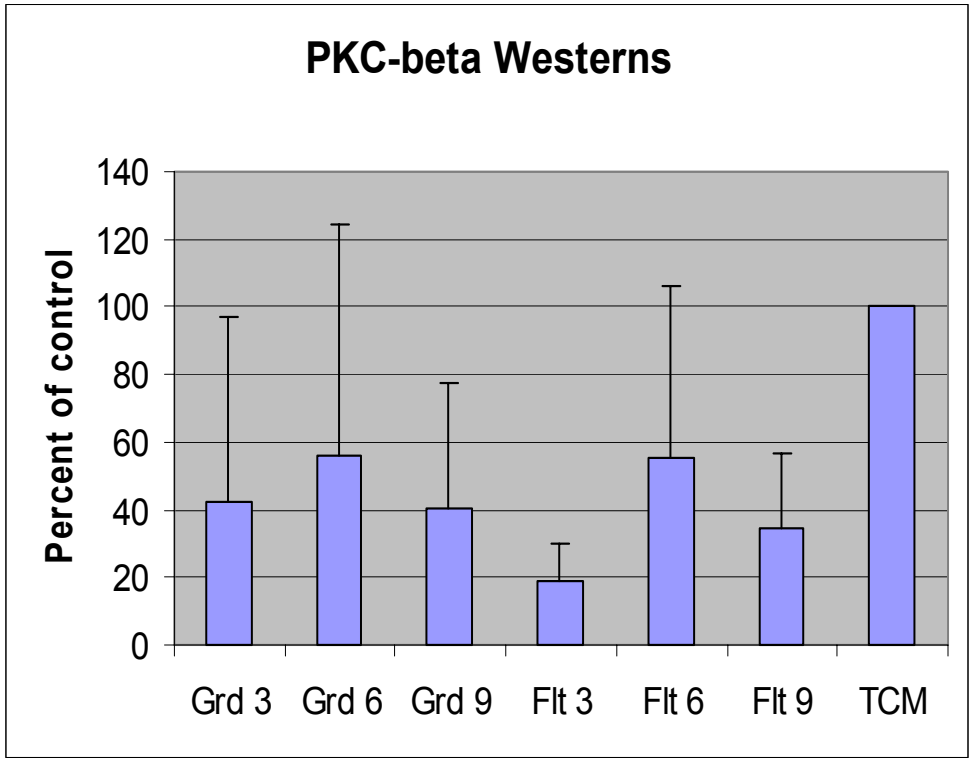
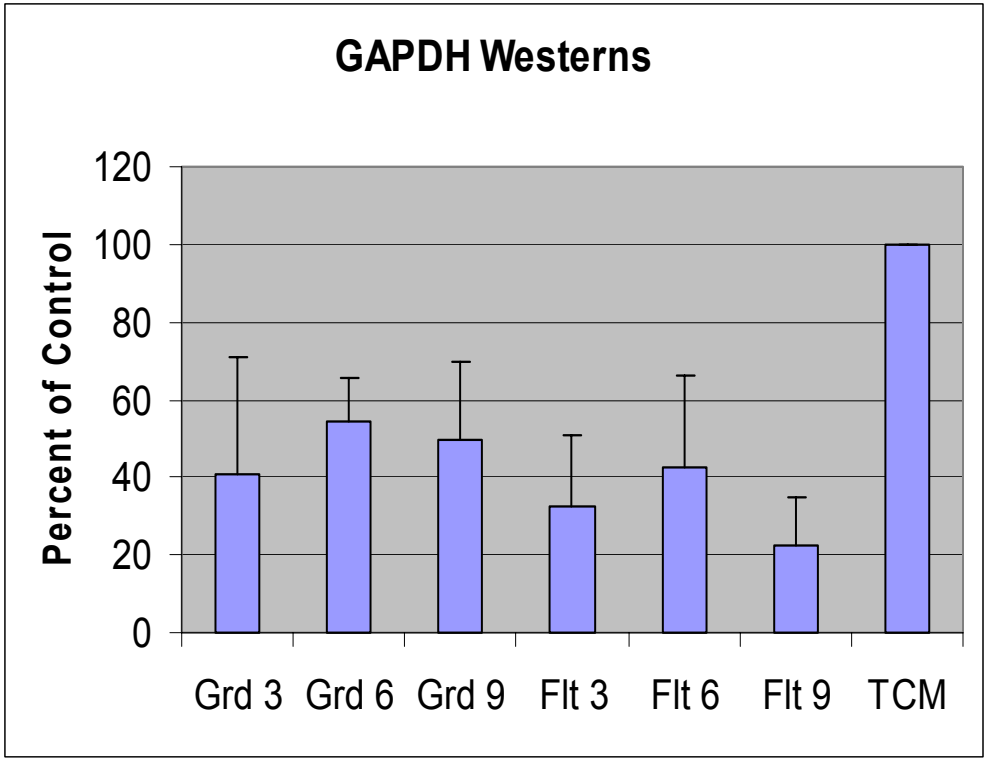


Figure 8 - Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody stained blot

SDS/PAGE gels prepared with equal loading of protein were blotted and stained with anti-GAPDH as the first antibody. The graph below demonstrates the average of four blots. An example of one of the blots is shown below the graph, where 6 µg/lane was loaded and the GAPDH gave a molecular weight of 42-44 kD.



CONCLUSIONS:

- Protein recovered from RNA*later* treated cells could be processed by SDS-PAGE and stained with protein stain.

- Protein could also be recovered from RNA*later* treated cells that had the RNA removed by an Ambion RNAqueous kit, using trichloroacetic acid precipitation.

- HRCE cells that were grown in the CBOSS equipment on the ISS during Expedition 3 and their ground control cells were shown to contain antigenic protein after treatment with RNA*later* and removal of RNA.

- Although on a protein per protein basis, the RNA*later* cells demonstrated slightly less antigenic protein than the untreated cells, still there was a high percentage of recovery of antigenic protein using three different antibodies in both the ground and flight samples.

REFERENCES

1. Rodrigo, MC, Martin, DS, Redetzke, RA, and Eyster, JM (2002) J Pharm and Tox Methods. 47:87-92.
2. Cowger,NL, Benes, E, Allen,P and Hammond TG (2002) J Appl Physiol 92:691-700.
3. Bicinchoninic acid (BC) protein assay kit procedure #TPRO-562, Sigma
4. Laemmli, UK (1970) Nature (London) 227:680-85.
5. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl, Acad. Sci, USA 76:4350-54.
6. Johnson, David, et al (1984) Gene Anal Techn. 1:3-8