Conducting On-orbit Gene Expression Analysis on ISS: WetLab-2

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• Provide a system that has the capability to process and analyze samples from various sources and can provide, as its end point, gene expression information to PIs in the form of on-orbit quantitative real-time PCR data.

• System can be either an integrated system or made up of separate modular components.
Concept of Operations

Animal Samples from Dissections or Cold Stowage

Cell culture or Microbial cultures

Tissue Disruption

Sample Prep

qRT-PCR

ISS Operations/Data Management

ARC Data Verification/Archive

Descent

RNA

Stabilized RNA

Examples:
• BIOS System (Ames)
• CGBA Science Inserts (BioServe)
• Biomodule (Bioserve)
• CellCult (BioServe)
• ADF Cell (TechShot)
• BSTC (JSC)
• RWPS (JSC)
• ESA or JAXA Experiment Container
Concept of Operations

Animal Samples from Dissections or Cold Stowage → Sample Prep

Cell culture or Microbial cultures → Sample Prep

Tissue Disruption → Sample Prep

qRT-PCR

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Science Stakeholder/Researcher

Examples:
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- ESA or JAXA Experiment Container
Constraints

Works in microgravity or can be easily modified to do so

Low power consumption

Low heat output

Low noise levels during operation

Must have flexibility over primer and probe selection

Preferences

LED light source

Few moving parts

Can be modified to fit in single locker
Instrument Selection

Cepheid SmartCycler

Modular Design

- Total of sixteen modules
- Modules can be independently programmed and run
- If one module fails, other modules still function
- Configurable within ISS rack volume constraints

Few moving parts (small fan in each module)

Rapid run time

Full flexibility over primer and probe selection and use

Multiplex capability: four fluorescence channels per module

Selection of a standard vs. automated instrument = we need to design a sample prep system
Sample Prep Constraints

**Crew Time**
- Whenever possible minimize the necessary crew manipulations
- Keep crew operations as simple as possible

**Cold Stowage**
- If possible, reagents should be long lived
- If possible, reagents will not require storage at refrigerated or frozen temperatures

**Hazardous chemicals**
- Limit Toxicity of chemicals
- If possible, avoid alcohols

**Containment**
- Plan to provide two levels of containment during sample preparation
- Plan to provide one level of containment during qPCR
Sample Prep Development

Initial approach: design system that extracts RNA from 2-3 common sample types (mouse, yeast, \textit{E. coli})

RNA must be of high enough purity/quality for qPCR

Pursuing two methods

Simplified RNA purification

- Column based purification with minimal number of steps and complexity
- Advantages: cleaner, works with intronless genes (more sample types), can return excess pure RNA to PI for ground studies
- Disadvantages: more steps, less simple

One Step Lysis

- Reagent added to cells to lyse then crude lysate is directly used in qPCR reactions
- Advantages: very simple procedure, few steps
- Disadvantages: many impurities in mix, may not work with some organisms and intronless genes
Commerially available
Simplified column based RNA purification procedure
Small components
No spin columns, syringe based
OmniLyse cartridge capable of lysing yeast cells and *E. coli*
RNA was of sufficient quality for RT-qPCR
System tested successfully with yeast and *E. coli*
Company worked with us to optimize the protocol for our needs
Final Optimized Procedure

Turn on OmniLyse unit (not needed for mammalian cells)

Lyse sample and bind RNA to column
  Performed by passing culture back and forth through OmniLyser and column
  Lysis buffer contains Proteinase K

Wash column
  Wash buffer contains Proteinase K

Wash column again
  Wash buffer does not contain Proteinase K

Purge column with air

Elute RNA
Prototype of closed system with syringes, valves and tubing was developed and tested

**Procedure**

All liquids (except sample) are preloaded in module
Sample is introduced into module using Transfer Tool
Activate OmniLyse unit and push sample back and forth
As it is lysed sample moves through the column and the RNA binds
Column is washed twice with wash buffers
Column is purged with air
RNA is eluted with elution buffer
RNA purified using prototype SPM shows compatibility with qRT-PCR.
Eluted RNA in syringe

Mix with qPCR reagents and primers and probes

qPCR reagents (enzymes, buffer, etc.) will be lyophilized

Location of reagents is not finalized – mix after elution or in Reaction Tube

Primers and Probes must be located in the Reaction Tubes

Remove air bubbles

Load Tubes
Procedure:
Syringe containing eluted RNA from the SPM is connected to the RAM
RNA may be mixed with lyophilized Reagent Mix (MM)
Mixture is pushed through line designed to remove air bubbles
Mixture is loaded into Repeater pipetter tip from the Wet Lab Kit
Repeater pipettor is removed and used to load the Reaction Tubes
Procedure:

Tubes have been modified to have a permanent cap with a pierced septa.
Reaction Tubes will contain dried DNA primers and probes.
Repeater pipettor containing RNA from RAM is used to manually load Reaction Tubes.
Reaction Tubes are loaded through septum.
Liquid must be located in the detection window.

Will use centrifugation.

RAM and tube load procedure are compatible with qPCR.
SmartCycler has been modified to fit in one EXPRESS Rack Locker
Drawer design – Unit will be pulled out to load tubes
Connected to dedicated laptop during entire run
When run is complete, data will be down-linked to the ground
Fits 16 reaction tubes
Future Work

Select reagent lyophilization provider
  Define location of qPCR reagents

Long term testing
  Stability of lyophilized qPCR reagents
  Stability of SPM reagents

Further testing on sample processing
  Test with more sample types (SWG)
  Test using low abundance RNA

Integrated testing
  Continue testing with prototype and final RAM and SPM versions
  End-to-end runs of experiments using hardware
Verification Flight Plan

Goal of Verification Flight:

On-orbit test and check-out of the WetLab-2 system in a systematic way to ensure it will return valid data to future researchers

Objectives of Verification Flight

Install hardware and software (Session 1)

Does real-time PCR data produced on-orbit match data on earth? (Session 2)
  • No convection or other issues

Does the Sample Processing Module function correctly on-orbit? (Session 3)
  • All fluidic manipulations function properly
  • Prove out system with first sample type

Does the Homogenizer unit function correctly on-orbit? (Session 4)
  • All fluidic manipulations function properly
  • Prove out system with second sample type

Flight results from each session will be compared to those obtained from the ground controls

Ground controls will be run with a 2-24 hour delay from the flight samples
### SmartCycler Characterization

#### Fluorophores

<table>
<thead>
<tr>
<th>Fluorophores</th>
<th>Channel</th>
<th>h Exc/Em</th>
<th>Calibrated</th>
<th>Tested</th>
<th>Crosstalk</th>
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<tr>
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<td>1</td>
<td>495/519</td>
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<td>Yes</td>
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<tr>
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<td>2</td>
<td>550/570</td>
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<tr>
<td>TET</td>
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<td>521/536</td>
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<td>No</td>
<td>N/A</td>
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<tr>
<td>Alex 532</td>
<td>2</td>
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<td>No</td>
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<tr>
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<tr>
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<td>Alexa 647</td>
<td>4</td>
<td>650/665</td>
<td>Yes</td>
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</table>
Promega GoTaq® Probe 1-Step RT-qPCR chosen

- Master Mix that includes all reagents needed for Reverse Transcription and qPCR
- Five-Six logs of dynamic range (mouse)
- Four-Six logs of dynamic range (yeast and *E. coli*)
- Company willing to make glycerol free reagents for lyophilization
- Test batch of glycerol-free reagents showed same activity/characteristics
- Promega has working relationship with our Lead lyophilization vendor

Shrimp DNase chosen as an additive

- dsDNA-specific DNase capable of removing residual genomic DNA
- Compatible with Promega Probe 1-Step RT-qPCR system
- Effectively removed residual genomic DNA as effectively as on column DNase 1 digestion
Shrimp DNase is as effective as on column DNase 1 treatment in removing carryover genomic DNA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ct</th>
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<tbody>
<tr>
<td>ClaremontBio (CB)</td>
<td>18.36</td>
</tr>
<tr>
<td>CB + Shrimp DNase</td>
<td>21.84</td>
</tr>
<tr>
<td>CB + Qiagen clean (Q)</td>
<td>16.82</td>
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<tr>
<td>CB+Q + Shrimp DNase</td>
<td>20.91</td>
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<tr>
<td>CB+Q + DNase 1 digestion</td>
<td>21.22</td>
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</table>
SmartCycler Characterization
Volume Effect

The upright volume test indicated a Ct dependence on volume. Ct values decreased as volumes increased from 20 to 50 uL. As volumes increased further from 50 uL to 100 uL, Ct values increased (Blue diamond line).

However, the same volume dependence was not seen in the inverted configuration. (Red square line)

Ct values from extended volume testing with Promega RT-PCR kit

<table>
<thead>
<tr>
<th>Volume (uL)</th>
<th>Ct</th>
<th>Volume (uL)</th>
<th>Ct</th>
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<tbody>
<tr>
<td>25</td>
<td>19.89</td>
<td>65</td>
<td>19.43</td>
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<td>35</td>
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<td>45</td>
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<td>85</td>
<td>22.35</td>
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<tr>
<td>50</td>
<td>16.84</td>
<td>95</td>
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<td>55</td>
<td>18.28</td>
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<td>23.44</td>
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### SPM-9/10 Testing on Three Model Organisms

<table>
<thead>
<tr>
<th>Sample Process</th>
<th>Organism</th>
<th>Elution Volume (ul)</th>
<th>Conc. ng/uL</th>
<th>Total Yield (ugs)</th>
<th>RIN</th>
<th>Ct (100ngs Template)</th>
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<tbody>
<tr>
<td>Manual</td>
<td>Yeast</td>
<td>220</td>
<td>36</td>
<td>7.2</td>
<td>5-7</td>
<td>26.6</td>
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<tr>
<td>SPM</td>
<td>Yeast</td>
<td>750</td>
<td>~6</td>
<td>4.5</td>
<td>5-7</td>
<td>27</td>
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<tr>
<td>Manual</td>
<td><em>E. coli</em></td>
<td>250</td>
<td>88.6</td>
<td>22.2</td>
<td>9-10</td>
<td>21</td>
</tr>
<tr>
<td>SPM</td>
<td><em>E. coli</em></td>
<td>300</td>
<td>110.2</td>
<td>33</td>
<td>9-10</td>
<td>20.2</td>
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<tr>
<td>Manual</td>
<td>MLO-Y4</td>
<td>220</td>
<td>50</td>
<td>11</td>
<td>5-7</td>
<td>25.3</td>
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<tr>
<td>SPM</td>
<td>MLO-Y4</td>
<td>1,100</td>
<td>15</td>
<td>16.5</td>
<td>1-2.5*</td>
<td>26.65</td>
</tr>
</tbody>
</table>

* = Needs improvement