Message

From: Ian Gibbons [/O=ATHERANOS ORGANIZATION/OU=FIRST ADMINISTRATIVE GROUP/CN=RECIPIENTS/CN=IGIBBONS]
Sent: 2/18/2010 7:38:39 PM
To: Sunny Balwani [/O=ATHERANOS ORGANIZATION/OU=FIRST ADMINISTRATIVE GROUP/CN=RECIPIENTS/CN=Sbalwani]; Elizabeth Holmes [/O=ATHERANOS ORGANIZATION/OU=FIRST ADMINISTRATIVE GROUP/CN=RECIPIENTS/CN=Eholmes]
CC: Gary Frenzel [/O=ATHERANOS ORGANIZATION/OU=FIRST ADMINISTRATIVE GROUP/CN=RECIPIENTS/CN=Gfrenzel]
Subject: System 4.0 PPT
Attachments: System 4.0.v2.ppt

As requested ...
System 4.0
System component requirements and selection

02/18/2010

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Overview

System 4.0 will be capable of performing any measurement required in a distributed test setting.

It is envisaged that several distinct measurement technologies will be incorporated.

The system will be broadly based on the existing cartridge and reader concepts.

Open architecture for both reader and disposable.

The number of total measurements per sample will be increased by 2 to 3-fold (target: 15 assays?)
Assay Menu

- It is crucial to further refine/define which sample types/assays/design requirements are important
- In the field of immunoassay we have good information and experience
- In other fields, we have less secure information
- Specifically:
  - Which nucleic acid analytes?
  - What is the sensitivity requirement?
  - How many and which cell types and surface markers?
Candidate technologies

ELISA using chemiluminescence (current)
ELISA using absorbence
General chemistry using absorbence
PCR and RT-PCR using fluorescence readout
Cell marker assays using laser fluorescence + movement of detector relative to cells
Electrochemistry for electrolytes and blood gases
Sample imaging using a camera (see Appendix 3)
General system requirements

- Sample: Blood, plasma and control materials (other types?)
- Sample processing: Plasma from blood, Lysis of cells
- Sample volume: < 20 uL (preferably < 5 uL)
- (For some purposes (e.g. very high multiplex), volumes as high as 200 uL may be permitted.)
- Assay menu: All assays available for system 3.0/3.1 + TBD analytes in other assay classes
- Size, weight: TBD but not larger then System 3.0
- Assay times: TBD but not longer than System 3.1
- Other capabilities: TBD but not less than System 3.0
Proposal for the basis of System 4.0

- Review available technologies: Done
- Define requirements
  - Needs versus wants
- First pass proposal
  - Based on current platform concept (cartridge + dispense)
  - Integrate the optical detection means in one low sample volume device
    - Luminescence
    - Absorbence
    - Fluorescence
  - Add a separate system for cell counting
  - Add red cell removal technology
  - <Add sample integrity evaluation means>
  - Chemistries proposed all exist
- Engineering review
### Comparison of requirements, capabilities and limitations of available detection technologies

<table>
<thead>
<tr>
<th>Technology</th>
<th>Analytes</th>
<th>Sensitivity</th>
<th>Dynamic range</th>
<th>Detector Difficulty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>1,2,3,4,5,6,7</td>
<td>High</td>
<td>30 fold</td>
<td>Low</td>
</tr>
<tr>
<td>Absorbance spectroscopy</td>
<td>1,2,3,4,5,6,7</td>
<td>High</td>
<td>30 fold</td>
<td>Moderate</td>
</tr>
<tr>
<td>Turbidimetry</td>
<td>1,3,5</td>
<td>Low</td>
<td>30 fold</td>
<td>Low</td>
</tr>
<tr>
<td>Nephelometry</td>
<td>1,3,5</td>
<td>Moderate</td>
<td>100 fold</td>
<td>Low</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>1,2,3,4,5,6,7</td>
<td>High</td>
<td>1000 fold</td>
<td>Moderate</td>
</tr>
<tr>
<td>Fluorescence spectroscopy</td>
<td>1,2,3,4,5,6,7</td>
<td>High</td>
<td>1000 fold</td>
<td>High</td>
</tr>
<tr>
<td>Luminometry</td>
<td>1,2,3,4,5,6,7</td>
<td>V. High</td>
<td>10,000 fold</td>
<td>Low</td>
</tr>
<tr>
<td>Cell counting</td>
<td>9</td>
<td>Good</td>
<td>100 fold</td>
<td>Moderate</td>
</tr>
<tr>
<td>Cell imaging</td>
<td>9,10</td>
<td>Moderate</td>
<td>30 fold</td>
<td>High</td>
</tr>
<tr>
<td>PCR (and RT-PCR)</td>
<td>8</td>
<td>V. High</td>
<td>10,000 fold</td>
<td>Moderate</td>
</tr>
<tr>
<td>Electrochemistry</td>
<td>11, others?</td>
<td>Moderate?</td>
<td>?</td>
<td>Low</td>
</tr>
</tbody>
</table>

#### Analyte class

<table>
<thead>
<tr>
<th>Key</th>
<th>Analyte class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Biomarkers</td>
</tr>
<tr>
<td>2</td>
<td>Microbial antigens</td>
</tr>
<tr>
<td>3</td>
<td>Small molecules (drugs)</td>
</tr>
<tr>
<td>4</td>
<td>Small hormones</td>
</tr>
<tr>
<td>5</td>
<td>Antibodies</td>
</tr>
<tr>
<td>6</td>
<td>Metabolites</td>
</tr>
<tr>
<td>7</td>
<td>Enzymes</td>
</tr>
<tr>
<td>8</td>
<td>Nucleic acids, viral genomes</td>
</tr>
<tr>
<td>9</td>
<td>Cell surface markers</td>
</tr>
<tr>
<td>10</td>
<td>Intracellular markers</td>
</tr>
<tr>
<td>11</td>
<td>Electrolytes and blood gases</td>
</tr>
</tbody>
</table>
## Comparison of Physical Technologies used for Assays

<table>
<thead>
<tr>
<th>Physical Technology</th>
<th>Application</th>
<th>Mechanical Difficulty</th>
<th>Chemistry Difficulty</th>
<th>IP/Licensing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separation</td>
<td>IA</td>
<td>Moderate</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Non-separation</td>
<td>Clinical chemistry</td>
<td>Low</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Non-separation</td>
<td>Nucleic acids</td>
<td>Low</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Non-separation</td>
<td>Immunochemistry</td>
<td>Low</td>
<td>High</td>
<td>Required</td>
</tr>
<tr>
<td>Physical multiplex</td>
<td>Any</td>
<td>Moderate</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Chemical multiplex</td>
<td>Immunochemistry</td>
<td>Low</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>Flow/Movement</td>
<td>Cell counting</td>
<td>Moderate</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Imaging</td>
<td>Cell counting</td>
<td>Low</td>
<td>Low</td>
<td></td>
</tr>
</tbody>
</table>
Separation of red cells and washing

- There is a trade-off between simplicity of operation of cartridge and instrument versus requirements for (1) low sample volume, (2) using plasma as the sample and (3) washing the capture surface in immunoassays

- We were not able to obtain a sufficient plasma sample from blood for multiplexed immunoassays

- Solutions to this problem are:
  - (1) Use blood as the sample
    - Calibration becomes more complicated
  - (2) Use lysed blood as the sample
    - Calibration should be corrected for HCT
  - (3) Separate plasma and use a very low volume measurement technology (e.g. Nanodrop)
    - Will require reverse engineering and/or a license
Special problem for blood samples
Red cell separation means

- Glass fiber wick or pad + plasma extraction
  - Vogel patent has expired
- Magnetic particle agglutination
  - ...
  - ...
- Pump through a frit??
  - ...

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Separation versus non-separation technologies

- Theranos has used separation-based ELISA as core technology. This requires efficient washing of the assay capture surface to remove unbound label (time consuming).
- There are several immunoassay techniques that have non-separation technologies
  - Typically these are:
    - Proprietary (license would be needed)
    - Fast
    - Not as sensitive as separation methods
- There are three such technologies that are very sensitive and general
  - EMIT (small molecule drugs) [Syva now Siemens]
  - β-Galactosidase complementation (Biomarkers, receptors etc.) [DiscoveRx]
  - Alpha screen (General immunoassay) [Packard]
- These are reviewed in Appendix 1
Compatibility and conflict between requirements for the different measurement technologies

- Each distinct detection technology compounds system (instrument and disposable) complexity and cost
  - Is a three-technology system 3x or 9x more difficult to develop?

- Temperature control
  - Thermo-cycler may compromise temperature control for the rest of the instrument

- Light shielding
  - Luminescence detector needs protection from high intensity light sources

- Time factors
  - Current Biomarker assay chemistry takes (say) 30 m
  - RT-PCR may take > 2 hours
Trade-off of sensitivity and time

- Luminescence ELISA can generally be complete in 15-30 m
- High sensitivity absorbance ELISA may need hours for equivalent sensitivity
- PCR sensitivity increases with # of cycles
  - 10 cycles: $10^3$-fold
  - 20 cycles: $10^6$-fold
  - 30 cycles: $10^9$ fold
Detection configurations compared (imaging not shown)

- UV/Visible spectroscopy
- Reflectance spectroscopy
- Turbidimetry
- Nephelometry
- Fluorescence spectroscopy
- Cell counting
- Electrochemistry

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Requirements, capabilities and limitations of candidate detection technologies and chemistries
ELISA with Chemiluminescence readout

- Very sensitive with very wide dynamic range
- Capable of giving results rapidly
- Suitable for *immunoassay* of small and large analytes
- Weakness is that reagent stability (E-Ab) and substrate contamination are crucial

- Essential technology
Absorbance

• Many applications
  • General chemistry (e.g. cholesterol)
  • Hemoglobin (and calculated HCT)
  • Albumin and total protein
  • TDM by licensed assays (EMIT/DiscoveRx etc.)
  • Enzyme assays (e.g. ALT)
  • ELISA with absorbance readout
    • Enables adaptation of existing assays?
  • Electrolytes (e.g. Ca2+)
    • Sensitive with moderate dynamic range
    • Take longer than those with chemiluminescent readout

• For full value wavelength range of 340 – 650 needed

• Essential technology
Fluorescence

- Essential for nucleic acid assays
- Not needed for clinical chemistry or immunochemistry
- Good sensitivity and dynamic range
- Capable of multiplexing

- Requires a high quality light source (laser[s])
- Compensation for light source instability is needed
PCR and Reverse Transcriptase-PCR

- **Essential** for all nucleic acid assay targets
- Requires both elevated temperatures (90°C) and temperature cycling
- Likely to require development of new disposable elements/surface chemistry
- Needs fluorescent readout
Cell counting

• Questions:

• How many markers?
  • >> How many lasers and complexity of the optics
  • One laser gives three colors

• Is rare cell detection needed?
  • Problem is sample volume and the need for cell concentration prior to detection
Rare cell analysis

• Circulating cancer cells
• Fetal cells in maternal blood

• Problem:
  • Cell numbers may be as low as (say) 10/mL
  • Blood drop = 20 uL
  • Cells/drop = 0.2: Impossible to detect reliably

• Solution is to pre-concentrate target cells from (say) 1 mL of blood then to detect
  • Not compatible with POC context
  • Technically demanding
Technologies deemed unnecessary

Reflectance
Imaging
Electrochemistry
Attractive potential add-on technologies
(See appendix 1)

- Non-separation, receptor-based assays with absorbance or luminescence readout
- Pulse oximetry
Review of issues for technologies not yet familiar at Theranos
Nucleic acid assays

- Polymerase Chain reaction (PCR)
  - Amplify DNA: 30 cycles = $10^9$-fold amplification
  - Needed for sensitivity

- Reverse transcriptase (RT-PCR)
  - Measure RNA (transcribe to DNA then apply PCR)
  - Needed for RNA virus detection

- Real time PCR
  - Semi quantitative

- All need temperature cycling and elevated temperatures
- Read-out by fluorescence
### PCR and RT-PCR:

**Time and temperature requirements, typical**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature, C</th>
<th>Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature</td>
<td>95</td>
<td>3</td>
</tr>
<tr>
<td>Anneal</td>
<td>65</td>
<td>1</td>
</tr>
<tr>
<td>Elongate</td>
<td>72</td>
<td>3</td>
</tr>
<tr>
<td>Cycle</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 (10^6 fold amplification)</td>
<td>140</td>
</tr>
</tbody>
</table>
Real-time PCR (TaqMan)
One favored readout means (needs a license)
Real-time PCR read-out

![Amplification - IFN gamma MP240399](chart.png)
Cell counting: option 1

Microvolume Laser Scanning Cytometry Platform for Biological Marker Discovery
Ian D. Walton, Louis J. Diiez, Gary Fraenkel, Jerry Chen, Jim Winkler, Scott M. Norton,
Aaron B. Kaiser
SunMed Inc., 1060 East Meadow Circle, Palo Alto, CA 94303.

- Spatial array of stained cells
- Raster optics (move laser)
- Or, image
- Simple capillary cartridge (shown for analysis of 32 samples)
Cell counting: Option 2

- Needs controlled flow
- Measure pulses over time
Questions/Issues

Complexity versus cost and practicality
Nanodrop does not measure absorbance and fluorescence in the same unit
Laser: size/cost versus # of cell surface markers

Costs
Do we need electrochemistry?

- Many analytes measured by electrochemical methods (O2, CO2, HCO3-, Ca2+, Mg2+) are only needed in emergency situations.

- Many analytes such as K+, Ca2+, Mg2+, Phosphorus (PO4^{2-}) and pH can be measured by absorbence.
  - Protocols are simple
    - Two or three liquid reagents
    - Mix with sample and incubate
Small volume assay reader concept

02/18/2010
Development Team

This presentation and its contents are Theranos proprietary and confidential
Strategic Issues For POC Assay Systems

- Performance equivalent to laboratory methods
- Multiplexed assay capability
  - Multiple assay type capability
    - Immunoassays
    - Direct assays (cholesterol etc.)
    - Enzyme assays
    - Nucleic acid assays
    - Electrolytes (and blood gasses??)
- Simplicity and reliability of use
  - Non-technical users
  - No false results
- Small sample volume
  - < (say) 5 uL blood
  - No hematocrit effect
- Speed
  - < (say) 15 m
- Ease of assay development
- Speed of assay development
- Low cost
  - Instrument
  - Disposable

*Italics emphasize possible improvements/extension on/off current system*
Small volume assay concept

- Blood sample: say 10 uL (= about 5 uL plasma)
- Sample capillary sucks plasma (say 3 uL; note filter does not need to be very efficient) from a (say) glass fiber filter.
  - Could also use a frit in a "tip" (?) + aspiration
- Plasma fills capillary and is then displaced into a dilution well. Mixing by repeated aspiration and re-expression >> (say) 30 uL diluted plasma
- Diluted sample (3 uL) is aspirated into standard Theranos tips (up to 8 assays = 24 uL diluted sample)
- Process assays with color forming chemistry (ELISA) generating (say) 3 uL colored product
  - Tip precludes evaporation
- Read in Nanodrop-style photometer

Advantages
- HRP chemistry enabled
- Small sample volume
- Red cell removal would be possible
- Higher multiplex might be possible
- Can also be used for clinical chemistry assays
  - Glucose, Cholesterol
  - Enzymes (ALT)

Disadvantages
- Light source required (can be very simple and inexpensive as can the detector, however)
- Need to wash sample chamber (Nanodrop cleans up fine by just wiping +/- one wash)
“Nanodrop” spectrometer

- Designed to measure protein and nucleic acid concentrations in very small volumes
- 200 – 800 nm
- 0.5 – 5 uL
- Measurement complete in a few seconds
Use of Nanodrop

Pipette (say) 2 uL onto pedestal

Instrument automatically defines a precise optical pathlength (1 mm)
## Instrument Specifications

**NanoDrop 2000/2000c – pedestal mode**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument Type:</td>
<td>Spectrophotometer</td>
</tr>
<tr>
<td>Minimum Sample Size:</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Pathlength:</td>
<td>1 mm (auto-ranging to 0.05 mm)</td>
</tr>
<tr>
<td>Light Source:</td>
<td>Xenon flash lamp</td>
</tr>
<tr>
<td>Detector Type:</td>
<td>2048-element linear silicon CCD array</td>
</tr>
<tr>
<td>Wavelength Range:</td>
<td>190-840 nm</td>
</tr>
<tr>
<td>Wavelength Accuracy:</td>
<td>±1 nm</td>
</tr>
<tr>
<td>Spectral Resolution:</td>
<td>≤1.8 nm (FWHM @Hg 253.7 nm)</td>
</tr>
<tr>
<td>Absorbance Precision:</td>
<td>0.002 absorbance (1 mm path)</td>
</tr>
<tr>
<td>Absorbance Accuracy:</td>
<td>±2% (at 0.76 absorbance at 257 nm)</td>
</tr>
<tr>
<td>Absorbance Range:</td>
<td>0.02 -300 (10 mm equivalent)</td>
</tr>
<tr>
<td>Detection limit:</td>
<td>2 ng/µL dsDNA</td>
</tr>
<tr>
<td>Maximum Concentration:</td>
<td>15,000 ng/µL (dsDNA)</td>
</tr>
<tr>
<td>Measurement Time:</td>
<td>&lt; 5 seconds</td>
</tr>
<tr>
<td>Footprint:</td>
<td>14 cm x 20 cm</td>
</tr>
<tr>
<td>Weight:</td>
<td>2.0 kg</td>
</tr>
<tr>
<td>Sample pedestal Material of Construction:</td>
<td>303 stainless steel and quartz fiber</td>
</tr>
<tr>
<td>Operating Voltage:</td>
<td>12 VDC</td>
</tr>
<tr>
<td>Operating Power Consumption:</td>
<td>12-18 W, (max 30 W)</td>
</tr>
<tr>
<td>Software Compatibility:</td>
<td>Windows® XP and Vista (32 bit)</td>
</tr>
</tbody>
</table>
Protein spectra

- Serial dilutions of a protein solution are shown
- Measurements are reproducible
- Spectral ratios make it easy to detect malfunctions (e.g. bubbles in the light-path)
Nanodrop applications illustrated

Performance measures
Sample volume independence
Protein assay (UV measurements)
Measurements in the visible
ELISA readout
Hemoglobin/HCT measurement
Dose-response is linear up to about 1.5 OD

Delta plot (280-500)

- $y = 0.3893x$
- $R^2 = 0.9983$
- $y = 0.3999x$
- $R^2 = 0.9996$

Target conc., mg/mL

OD280-OD500

- All data
- Omit OD > 1.2
- Linear (All data)
- Linear (Omit OD > 1.2)
Good linearity from low to high absorbance
Precision is good.
Even at low A values

<table>
<thead>
<tr>
<th>BSA, mg/mL nominal</th>
<th>A, 1em,280, avg</th>
<th>N</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.923</td>
<td>11</td>
<td>0.9</td>
</tr>
<tr>
<td>0.2</td>
<td>0.093</td>
<td>5</td>
<td>8.2</td>
</tr>
<tr>
<td>0.05</td>
<td>0.019</td>
<td>4</td>
<td>18.6</td>
</tr>
</tbody>
</table>

LOD (95% confidence) = 0.01 (Absorbance); 0.02 mg/mL (BSA conc.)
System also works in the visible range
System response is independent of sample volume
ELISA: Visible spectra

Helena Protein C Std. Curve: Detail showing replicates
ELISA assay comparison (signal)
Helena Protein-C assay

\[ y = 0.1586x - 0.0001 \]
\[ R^2 = 0.9981 \]
Protein-C ELISA response comparison

\[
N0 = 0.0048x^2 - 0.0518x^2 + 0.2338x + 0.0156
R^2 = 0.9954
\]

\[
M5 = 0.0231x^3 - 0.2833x^2 + 1.3986x + 0.1175
R^2 = 0.9895
\]
Hematocrit/Hemoglobin assay
Standard clinical assay for Hb (Drabkin’s method)
(RBCs are lysed; Hb is converted to Cyan:Met-HB; read A540 nm)
Possible applications in a next generation Theranos Instrument

- Colorimetric ELISAs
  - HRP or APase labels

- Clinical chemistry analytes can be measured colorimetrically
  - Glucose
  - Cholesterol
  - HDL-cholesterol
  - Electrolytes

- Enzyme and other types of assays with NADH or NADPH readout
  - EMIT! (TDM Assays!)
Signal is lower than for a standard spectrometer

- Path length is 1 mm
- A is about 10 – 20 % that of a MTP reader (path length 0.5 – 1.0 cm)

\[ \Delta A \text{ (SD) 0.002 } \text{i.e. CV = 10\% at A = 0.02 (OD 0.2)} \]
- This can/may be compensated for by letting signal (A) values rise into the range (say) 0.2 – 5.0
  - Elisa assays: let enzyme work longer and use elevated temperature
  - General Chemistry: dilute less
Nanodrop 3300: Spectroflorometer

Q: Is the Thermo Scientific NanoDrop 3300 an upgrade to the NanoDrop® 2000?
A: No, the NanoDrop 3300 is used to measure fluorescence whereas the NanoDrop 2000 is used to measure absorbance.
# Nanodrop Fluorimeter Specifications

## Specifications

<table>
<thead>
<tr>
<th>Specification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument Type</td>
<td>Fluorospectrometer</td>
</tr>
<tr>
<td>Minimum Sample Size</td>
<td>1 μl</td>
</tr>
<tr>
<td>Light Sources</td>
<td>3 light-emitting diodes (LEDs)</td>
</tr>
<tr>
<td>Excitation Maxima</td>
<td>UV: 365 nm, Blue: 470 nm, White: 460-480 nm</td>
</tr>
<tr>
<td>Detector Type</td>
<td>2048 - element linear silicon CCD array</td>
</tr>
<tr>
<td>Wavelength Range</td>
<td>400-750 nm</td>
</tr>
<tr>
<td>Wavelength Accuracy</td>
<td>1 nm</td>
</tr>
<tr>
<td>Spectral resolution</td>
<td>8 nm (FWHM at Hg 546 nm)</td>
</tr>
<tr>
<td>Fluorescence Precision</td>
<td>&lt;0.1% CV (10 mM fluorescein)</td>
</tr>
<tr>
<td>Detection Limit</td>
<td>&lt;1 femtometer fluorescein</td>
</tr>
<tr>
<td>Measurement Cycle Time</td>
<td>2 - 10 seconds</td>
</tr>
<tr>
<td>Dimensions (footprint)</td>
<td>14 cm x 20 cm</td>
</tr>
<tr>
<td>Weight</td>
<td>1.2 kg</td>
</tr>
<tr>
<td>Sample Pedestal Material of Construction</td>
<td>303 stainless steel and quartz fiber</td>
</tr>
<tr>
<td>Operating Voltage</td>
<td>5 Vdc (supplied by USB port, no external power supply)</td>
</tr>
<tr>
<td>Standby Power Consumption</td>
<td>2 W</td>
</tr>
<tr>
<td>Software Compatibility</td>
<td>Microsoft Windows® 2000, XP, and Vista (32 bit)</td>
</tr>
<tr>
<td>UL/CSA and CE</td>
<td>All units are approved to these standards</td>
</tr>
</tbody>
</table>
Nanodrop Fluorimeter: Sensitivity and dynamic range

Alexa 555 Linearity on the NanoDrop 3300

\[ y = 2.12x + 6.66 \]
\[ R^2 = 1.000 \]

Alexa 555 Linearity on the NanoDrop 3300
Low Concentrations

\[ y = 2.33x - 0.06 \]
\[ R^2 = 0.9991 \]
Question and Idea

- Why not adapt the Nanodrop spectrometer to chemiluminescence?
- Develop a multi-detection mode platform
  - Absorbence
  - Fluorescence
  - Luminescence
- Problem might be smaller volume >> less signal
- The Nanodrop optic, however, appears to use a larger fraction of the sample volume than conventional spectrometers (and the Edison?)
  - Edison light collection volume has been estimated as about 5 uL but only a fraction of the light from that volume gets to the PMT.
Current Instrument Light Collection

Glowing reaction product

PMT aperture
US 6,628,382 Sept. 30 2003 CW Robertson
Priority date: 08/1999

1. A photometric or spectrophotometric apparatus wherein a sample in the form of a liquid drop is contained by surface tension forces between two planar surfaces, one containing a photometric or spectrophotometric source and the other a photometric or spectrophotometric detector and an optical path is established through the sample between the two surfaces said apparatus comprising:

first and second anvil surfaces at least one being moveable relative the other to any one of three positions:

an adjustable sample loading position so selected that the at least one moveable surface and the other surface are so remotely spaced that a liquid drop can be placed on the first surface;

an adjustable sample compression position so selected that the surfaces are opposed and substantially parallel and proximally spaced so that the liquid wets and spreads upon both surfaces;

an adjustable sample measuring position so selected that the opposed substantially parallel surfaces are spaced apart to pull the sample into a column wherein it is contained by surface tension thereby providing an optical path for a photometric or spectrophotometric measurement.
NanoDrop: Definition of light path

Sample path length and illuminated volume are defined by the geometry of the pedestal.

Volume in excess over 1 uL is expressed out of the light beam and contained by surface tension.
Optics For Three Detection Technologies

(1) Absorption

Detector

Light source producing a wide highly-collimated beam

Defined illuminated volume
Optics For Three Detection Technologies
(2) Fluorescence

Light source producing a narrow highly-collimated beam

Detector

Defined illuminated volume
Optics For Three Detection Technologies

(3) Luminescence

Detector

Defined volume for light collection
Alternative low volume system for optical detection of fluorescence using optical fibers

See also: US 4,676,640 (expired)

US4676640.pdf
Another version of the fiber approach for low volume fluorescence detection and cell counting

See: US 4,676,640 (expired)

US4676640.pdf
What would be needed to incorporate this technology in our system?

- Interface with liquid dispensing
  - Demo in progress
- Interface with RBC filtration
- Add a simple light source to the instrument
- Detector
  - PMT? or simple CCD (spectroscopy [many wavelengths]) or Photodiode (one color)
- IP: license or (better) reverse engineer
Recommendations

- Selected candidate technologies
  - Luminescence
  - Absorbence
    - Spectroscopy needed (multi-wavelength)
    - Wavelength range 340-650 nm
  - Fluorescence
  - RT-PCR
  - Cell counting

- Need to consolidate optics around a low volume detection means (invention needed)
  - Nanodrop-like?

- Need to invent and develop red cell separation technology
Pipettor head multiplicity: Issues

• Holding liquid during incubations
  • “Tip” versus “well”
  • Gravity
    • Tip orientation
    • Small volume would likely remain fixed (even if tip is vertical)
  • Evaporation (very important if volume < 5 uL)

• Pressure transmission when picking up tip
  • Premature ejaculation

• Precise positioning
  • Nanodrop approach would require good precision in x, y and z
  • < 0.1 mm is OK and easy to implement
  • But multiple head compounds the problem

• Temperature control
  • Absolute requirement for immunoassays and PCR
Cartridge layout and number of assays

- Current design completely fills the footprint (close packing of assay elements)
- Footprint is dominated by diameter of reagent tubes and the tip boss
- Can these be smaller?
- Can some reagents be shared?
  - One head concept makes this easier
  - But, there is a time penalty
Engineering evaluation

- Feasibility review
  - Complete analyte list
  - Complete chemistry review against all analytes
  - Are integrated optics feasible?
  - How to implement more assays on same footprint
    - Can footprint be bigger?
    - Cartridge height could be less for smaller volume assays

- Development plan

- Costs and Development Time
Appendix 1

Some alternative technologies including non-separation methodologies

Review of Theranos requirements against possible licensable technologies
Theranos mission
Instrumented system

To enable rapid, accurate, multiplexed POC measurements of key biomarkers and therapeutic agents

To replace laboratory methods in monitoring disease and therapy
Capabilities (ideal)

Sample types
- Blood (F/W, venous), serum, plasma, urine

Marker types
- Proteins
- Small molecules
- Metabolites
- Nucleic acids
- Cell markers (Surface + Internal)
- Electrolytes

Number of multiplexed markers
- Up to 20

Number of assays available
- >100

Specificity, accuracy, precision and sensitivity
- Match current state of the art

Size and speed
- Match current POC state-of-art
  - Time < 18 min

Stability
- > One year at RT

Theranos cost per result
- < $3 equivalent to $18/sixplex; Price = > $100 at > 80% gross margin

Disposables/year
- > 1,000,000

FDA, CLIA cleared, ISO-9xxx
Patent protected
Sample integrity and volume measurement
Sample pre-treatment (including removal of red cells)
## Comparison of current Theranos and Competing technology

<table>
<thead>
<tr>
<th>Sample types</th>
<th>Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood (F/B, venous), serum, plasma, urine</td>
<td></td>
</tr>
</tbody>
</table>

**Marker types**
- Proteins
- Small molecules
- Metabolites
- N/O nucleic acids
- Cell markers (Surface + Internal)

**Number of multiplexed markers**
- Up to six

**Number of assays available**
- 100

**Specificity, accuracy, precision and sensitivity**
- Match current state of the art

**Size and speed**
- Match current POC state-of-art
  - Time < 15 min

**Stability**
- > One year at RT

**Theranos cost per result**
- <$3 equivalent to $18/sixplex
- Price = $100 at 80% gross margin

**Disposables/year**
- > 1,000,000

**FDA cleared**
- Match

**Patent protected**
- Match

**Worse (30-plex available)**
- Match

**Better**
- Match

**Worse**
- N/A

**Worse**
- 100

**Worse**
- ?

**Worse**
- ?

**Worse** (Situation improving this year)
<table>
<thead>
<tr>
<th>Attribute</th>
<th>Current</th>
<th>Possible</th>
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<tbody>
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<tr>
<td>• Blood (F/S, venous), serum, plasma, urine</td>
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<tr>
<td>Marker types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Proteins</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>• Small molecules</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>• Metabolites</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>• Ions (Ca2+ etc)</td>
<td>N/A</td>
<td>?</td>
</tr>
<tr>
<td>• NOT Nucleic acids</td>
<td>NO</td>
<td>? (NEW SYSTEM)</td>
</tr>
<tr>
<td>• Cell markers</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Number of multiplexed markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Up to six</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Number of assays available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 100</td>
<td>NO</td>
<td>YES</td>
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<td>Specificity, precision and sensitivity</td>
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<tr>
<td>• Match current state of the art</td>
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<td>Size and speed</td>
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<tr>
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<td>MAYBE</td>
</tr>
<tr>
<td>• Time &lt; 15 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stability</td>
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<tr>
<td>• &gt; One year</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Theranos cost per result</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• &lt;$3 equivalent to $18/sixplex;</td>
<td>NO</td>
<td>MAYBE</td>
</tr>
<tr>
<td>• Price = &gt;$100 at &gt;80% gross margin</td>
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<tr>
<td>Disposables/year</td>
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<td>Instruments/year</td>
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<td></td>
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<tr>
<td>• 1,000</td>
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<td>FDA, CLIA etc. cleared</td>
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</tr>
<tr>
<td>•</td>
<td>NO</td>
<td>YES</td>
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</tbody>
</table>

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## Existing technology capabilities

**Sensitivity limit (Can we do better for biomarkers? NO)**

<table>
<thead>
<tr>
<th>Calculations</th>
<th>Chemiluminescence EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Specific signal</strong></td>
<td></td>
</tr>
<tr>
<td>Antigen conc.</td>
<td>5.00E-01 pg/mL</td>
</tr>
<tr>
<td>Dilution</td>
<td>3.00E+00 fold</td>
</tr>
<tr>
<td>Diluted antigen conc.</td>
<td>1.67E-01 pg/mL</td>
</tr>
<tr>
<td>Efficiency of binding</td>
<td>1.00E+01 %</td>
</tr>
<tr>
<td>Enzyme molecules/antigen</td>
<td>2.00E+00 mole/mole</td>
</tr>
<tr>
<td>Enzyme turnover</td>
<td>5.00E+04 sec^{-1}</td>
</tr>
<tr>
<td>Enzyme conc.</td>
<td>3.33E-02 pg/mL</td>
</tr>
<tr>
<td>Interrogated volume</td>
<td>1.00E+06 ul.</td>
</tr>
<tr>
<td>Enzyme quantity</td>
<td>3.33E-05 pg</td>
</tr>
<tr>
<td>Enzyme MW</td>
<td>1.20E+05</td>
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<tr>
<td>Avogadro's #</td>
<td>6.04E+23</td>
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<tr>
<td>Vmax</td>
<td>8.39E+06 molecules of product/sec</td>
</tr>
<tr>
<td>S/K</td>
<td>5.00E-02</td>
</tr>
<tr>
<td>v</td>
<td>4.19E+05 molecules of product/sec</td>
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<tr>
<td>Quantum yield</td>
<td>5.00E-01 %</td>
</tr>
<tr>
<td>Photon production</td>
<td>2.10E+03 counts/sec</td>
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<tr>
<td>Detection efficiency</td>
<td>5.00E+01 %</td>
</tr>
<tr>
<td>Signal</td>
<td>1.05E+03 counts/sec</td>
</tr>
<tr>
<td>Enzyme molecules</td>
<td>1.66E+02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Background signal</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal</td>
<td>100 counts/sec</td>
</tr>
<tr>
<td>Signal</td>
<td>300 counts/sec</td>
</tr>
<tr>
<td>S/B</td>
<td>5.59E-01 NSB (best case)</td>
</tr>
</tbody>
</table>

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Some current potentially competing technology systems

The clinical laboratory ***
Service providers ***
Platforms for customer assay development
ELISA
  • Multiplexed Imaging (Searchlight)
Lateral flow (Biosite)
Electrochemiluminescence (MSD)
Time resolved fluorescence (TRF)
Non-separation methods
  • Alpha screen (Packard)
  • Enzyme complementation (DiscoveRx)
  • Homogeneous time-resolved fluorescence (HTRF)
  • EMIT (small molecules)
  • FP (small molecules)
  • Surface plasmon resonance ("Reagent-free", Biacore, Forte Bio)
  • Evanescent wave
FACS screen
  • Guava
Bead-based multiplexes (LumineX, BD etc.)
POC niche
  • Cholesterol (Lipids)
  • Abaxis (General chemistry + low sensitivity immunoassay)
  • Coaguchek etc. (PT)
  • Hemoccue (Hemoglobin)
  • Many (blood glucose)
  • Metrika (HbA1c)
  • Ions (ISE) > IA (I-Stat)
Existing competitive technology limitations

Factory calibration

Validation problems (multiplexes)

Sample handling (blood)
  • Stability

Sample volume (blood)
  • Too large
Potential improvements to our existing system

Up to 12 assays
Smaller sample 10 uL at 6-plex?
Faster (assays complete in < 15min?)
Smaller (1/4 th current size?)
More precise (< 5 %CV?)
More reliable (< 1/1000 failures?)
Add selected general chemistries
Add other types of marker
Candidate supplemental/alternate technologies

Examples
- DiscoveRx
- AlphaLISA
- EMIT
- HTRF
- ELAST
- ...
Luminescence Oxygen Channeling IA (LOCI, Alpha Screen)

Light (long $\lambda$)

Antibody 1 attached to Sensitizer-dye-doped particle

Particles close together

Add sample

Excited dye$^+$ + O$_2$ $\rightarrow$ O$_2^\cdot$
(Singlet oxygen)

No analyte in sample

O$_2^\cdot$

Radical decays with no light emission

O$_2^\cdot$

Many photons per antigen

O$_2^\cdot$

Excites fluor

Antibody 2 attached to Fluor-doped particle

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Technology comparison

Scaled units

Log [TNFα(g/mL)]

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81
Enzyme complementation (DiscoveRx) β-Galactosidase

Native β-Galactosidase polypeptide

Mutant, inactive β-Galactosidase polypeptide (EA)

Small N-terminal polypeptide (ED)

Enzyme Donor “ED” (Inactive)

Mix

Native enzyme is a tetramer

Complex (Active) forms quickly

Enzyme acceptor “EA” (Inactive)
Drug-labeled Enzyme Donor “ED” (Inactive)

Add sample + Anti-drug AB

Excess drug in sample
No drug in sample

Complex is active
Steric exclusion prevents complementation

Enzyme acceptor “EA” (Inactive)
(Add in excess)

No enzyme activity

Enzyme complementation immunoassay
(Non-separation, rapid)
EMIT (Enzyme multiplied immunoassay technique)
Rapid, non-separation measurement of small molecule drugs
(Siemens)

Protocol
Mix sample and two reagents.
Measure $\Delta A_{340}$
Assay is complete in about one minute

Anti-drug antibody
No drug in sample
Drug in sample
Enzyme-labeled drug hapten

(Drug)
$\Delta A_{340}$
HTRF

- "Homogeneous time-resolved fluorometry"
- No separation required

Fig. 1: HTRF principle with Eu²⁺ cryptate and XL665 as respectively donor and acceptor. When the two entities come into close proximity and upon excitation, FRET occurs and XL665 re-emits a specific long-lived fluorescence at 665 nm.
• Dissociation-enhanced lanthanide fluorescence immunoassay
• Europium chelate labeling
• Fluorescence enhanced by release of Eu and shielding on a micelle

Figure 1. Unique fluorescence properties of lanthanides. (a) long fluorescence decay times; (b) large Stokes’ shift.

Figure 2. Dissociation enhancement.
• ELISA amplification system
• Signal amplification
• Generally not effective because of background amplification (Contrast PCR)
Attributes (good and bad) of selected competing technology
Assay Protocols

- Block MSD MULTI-SPOT plate for 1 hour, wash
- Add 25 μL of assay diluent solution to each well
- Add 25 μL of calibrator or sample (undiluted for Panel I; 20-fold diluted for Panel II; 250-fold for PICP assay; and 20-fold for TGFß1 assay) to each well
- Incubate with shaking for 120 minutes, wash
- Add 25 μL of labeled antibody solution to each well
- Incubate with shaking for 60 minutes, wash
- Add MSD Read Buffer
- Read plate on MSD Reader
Chemical multiplexing

- Coded beads (Luminex, BD, BioRad)
- eTags (Monogram)
- The main problem is complexity and reliability
- In the case of chemical multiplexing background can be a problem
Luminex: Multiplex vs Singleplex

Lower Limit of Quantitation

- Multiplex
- Singleplex

Concentration (pg/ml)

0 2 4 6 8 10 12 14 16 18 20

IL-1b  IL-2  IL-4  IL-6  IL-8  IL-10  IL-12  IP-10  TNF-a

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Biosite

Multiplexed (3) IA
Good sensitivity
Blood samples (50 -200 uL)
Precision may not be good enough
Small menu
FDA cleared
Requirements for other types of assay

General chemistry: glucose, lipids
Enzymes: ALT etc.
Electrolytes and blood gasses
Extending the capability of the current system
Glucose assay chemistry

Current approach is chemiluminescent
• Compatible with current instrument
• Glucose + ATP > Glucose-phosphate + ADP (Hexokinase)
• ATP + D-Luciferin + O2 → Oxyluciferin + AMP + PPI + CO2 + Light (560nm) (Luciferase)
• Reverse reading assay, measures ATP remaining after ATP is converted to ADP

If this approach works
• Lipid assays and enzyme assays may be feasible
What additional critical analytes do we need/want?

Clinical chemistry
- Glucose (WIP)
- HbA1c (Method proposed; Ab availability is an issue)
- Lipids
  - Cholesterol, HDL-C, LDL-C, Triglycerides
  - ALT

White cell markers ??
- CD4, CD8 etc.

Small molecules with no IA chemistry compatible with current system and possible modifications
- Ions (detection means)
- Serotonin (organic chemistry required)
Appendix 2
Selected, existing POC systems for comparison
Biosite: Multiplexed Immunoassay
Cholestech: Multiplexed lipids and very limited other analytes
Abaxis: Multiplexed general chemistry and very limited immunoassay menu
HemoSense: PT/INR (only)
Hemocue: Hemoglobin and glucose
I-Stat: Multiplexed electrolytes and limited immunoassay
Pulse Oximeter

• Measures Hb saturation with O2
• Non-invasive
• Could easily be added/attached to our instrument
Appendix 3: Imaging Samples

Measurement of sample volume
Determination of sample integrity

02/18/2010

This presentation and its contents are Theranos proprietary and confidential
Imaging enables sample volume measurement

- Verification that the sample has the correct volume
- Verification that sample delivery was good
- Measurement of sample volume
  - Enables result correction for improper volume
- Inexpensive camera will work for this purpose
Calculation of volume (example)

Example 4: Geometry of measurement in a conical capillary (often used for tips)
Rb = radius at base of cone
L = length
L1 = distance from (projected) top of the cone to lower sample meniscus
L2 = distance from (projected) top of the cone to lower upper meniscus
Volume introduced = \( p^* \times \frac{(Rb/L)^2 \times [(L1)^3 - (L2)^3]/3}{3} \)
Tan \( \theta \) = Rb/L
Bubbles

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Images

B: Sample transfer device with its capillary filled with sample. The "fill to" location is indicated.

C: Sample transfer device with sample displaced by movement of the plunger.

D: Sample transfer device with sample incompletely displaced.
Measurement from image of conical capillaries

\[ y = -0.0002x^2 + 0.0252x + 0.3199 \]

\[ R^2 = 0.996 \]