To: Elizabeth Holmes[eholmes@theranos.com]; Sunny Balwani[sbalwani@theranos.com]
From: Mona Ramamurthy
Sent: Mon 9/9/2013 11:37:39 PM
Importance: Normal
Subject: FW: Summary Document
Received: Mon 9/9/2013 11:37:40 PM
Assay Development Strategy for Immunoassays.docx
SG_9-5-2013.docx

Per Elizabeth's voicemail, I spoke to Surekha this afternoon. She said she was almost done with the summary report and wanted to complete it while being here. Here it is. Please let me know if you think this is sufficient or she should add more. I can come by and discuss as well.

From: Surekha Gangakhedkar
Sent: Monday, September 09, 2013 2:53 PM
To: Mona Ramamurthy
Subject: Summary Document

Mona,

Please find attached the summary document we discussed. Let me know if you have any questions. I believe this captures all the necessary information and has the links for the project specific folders in the T drive and the J drive.

The file is also saved in the location - T:\Assay_Systems\Assay Development Reports\Assay Development Strategy for Immunoassays.doc

Also attached is the copy of my resignation letter that I had submitted to Elizabeth on 9/5/2013 for your reference.

Thanks,

Surekha

Assay Development Strategy for Immunoassays

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1. **Business Development will provide**

   a. Assay specs: Range, Sensitivity requirement, Matrix
   b. Determine gold standard: Information about Reference test

   Theranos assay range determined on the basis of information above , normal and pathological ranges.

2. **Antibody Selection**

   a. Order Monoclonal (preferred) and Polyclonal antibodies:
   Based on the availability of antibodies approximately 10 (if available) antibodies are ordered. Criteria based on Epitope, Purity, Presence of carrier proteins, Sufficient amount to conjugate (100 ug levels), Cost.

   b. Order analyte from 2 sources. Also order WHO standard, IRMM reference material if available.

   c. Antibody screen:

- For Sandwich ELISA, conjugate all the available antibodies both as Capture (Biotin) and Detection (Alkaline phosphatase) with 100 ug of antibody per conjugation reaction. For Competitive ELISA only biotin conjugation is required, since detection reagent will be analyte – Alkaline phosphatase conjugate.
- Screen each capture antibody against all available detection antibodies on a 384 well MTP, with a 4 - point standard curve comprising of the high, medium, low and no analyte levels.
- An initial capture antibody concentration of 10 ug/mL and Dab of 50 ng/mL would be a good place to start. These concentrations are based on ranges that normally yield good response for the sandwich assay format.
- Refer to assay protocols on the T drive/Assay group protocols folder for general procedures.
- Amongst the antibody pairs that yield a good dose response, pick 3 pairs with the highest Signal/Noise ratio.
- Compare the response of final 3 antibody pairs on the Edison with a 6-8 point standard curve in assay buffer, whole blood and plasma. The final choice for the antibody pair will depend on the response in whole blood and plasma. Criteria for the final pair will be good dose response, sensitivity, %CV in physiological matrix.
- Test for cross reactivity and interference with related analytes. Select antibody pair with no or minimal cross-reactivity.

3. **Assay Optimization** – on Edison and in final matrix (whole blood, plasma, serum).

- Optimize the assay for Capture antibody
  - Concentration: e.g. Titrate at 2.5, 5, 10, 20 ug/mL. Blocking buffer (TBS, 3% BSA, 0.05% NaN3) is used as the diluent.
  - Coating conditions: Standard blocking buffer is TBS, 3% BSA. Test other blocking buffers for improvement in dose response.
  - Coating time: Standard time for production is 30'/30'/10'.
- Optimize the assay for Detection antibody
  - Concentration
  - Diluents: Commercial stabilizers (Stabilzyme, Biostab,Stabilguard).
  - Conjugation ratios: use 50 and 200 ug of raw antibody during conjugation, compare to the standard 100 ug conjugation reaction to check for any improvement in sensitivity.
- Criteria for capture and detection antibody optimization are to improve the signal/noise ratio, sensitivity, better %CV.
- Assay time: Basic length of the assay is 10'/10'/10' (Analyte /Dab /Substrate incubation time). Test for different analyte and detection antibody incubation times.
- Finalize analyte dilution for cartridge. Basis for dilution factor will depend on sensitivity requirement for the assay, ranges and sensitivity requirements of other analytes on the multiplex panel.
- Effect of Post sample wash.
- Analyte validation: Test analyte on reference test and re-assign if needed.

4. **Verification /Validation**

- **Assay range/Quantitation limit**: Include analyte levels 2-fold above and below the range of the assay to confirm the ULOQ and LLOQ. % Recovery at these levels should be within 25% of nominal at the ULOQ and LLOQ. %CV should be less than 20% for the mid range and less than 25% for the high and low concentrations.
- **Precision tests**:

a) Validation controls (6 analyte levels will made in plasma spanning the range of the assay) will be tested in replicate cartridges (N=6) over multiple days. Precision data will be presented in concentration CV based on an assay buffer calibration curve. The acceptance criteria is that the average % bias and %CV (over 6 assays) must be less than 20% for the middle three concentrations and less than 25% for the high and low concentrations.

b) % CV across 24 cartridges (run on multiple instruments) at mid-range analyte should be less than 20%.

- **Specificity**: Spike analyte into whole blood and plasma at a minimum of 6 levels and calculate % recovery with respect to an assay buffer curve. % Recovery and % CV should be less than 20% at the mid range levels and less than 25% at high and low levels.
- **Selectivity**: applies to drug assays and analytes with low or undetectable levels.10 individual plasma (5 male 5 female) will be tested at LLOQ, below LLOQ and unspiked

levels. Samples will be run in triplicate cartridges. Calibration will be back calculated and must be within 25% of nominal to pass. The acceptance criteria for this experiment is that 8 out of 10 of the results from the individuals need to be within 25% of the nominal concentration for the LLOQ spike and 8 of 10 of the unspiked have to have results below LLOQ.

- **Dilution linearity** of analyte is tested in physiological matrix (plasma or serum). Clinical sample with a high level is serially diluted (2-fold recommended) with a sample with low or no detectable level of analyte. In the absence of availability of low samples, analyte is spiked into plasma or serum with known levels (preferably low) and serially diluted. Acceptance criteria for % recovery are as indicated above for the assay range and Precision tests.
- **Clinical correlation**: Test Clinical samples (N=20) for correlation of Commercial ELISA kit with Theranos assay.
- **Reference range**: Test N=20 of Normal healthy samples
- **Assay Extended range**: Test assay linearity and response at high concentrations (4x of ULOQ) of analyte.
- **Interfering substances:** Test impact on % recovery on assay in the presence of interfering substances –
- **Matrix effects**: % Recovery and % CV of a 6 point standard curve in Hemolysed plasma, Lipemic plasma are measured with reference to an assay buffer calibration curve. Acceptance criteria are same as above.

5. Initial Stability Tests in pre-built cartridges

- Capture antibody stability on Edison Tips.
  8 week stability of coated tips stored at 4C, RT. Time points to be tested at 0, 1, 2, 4, 8, 12 and 24 weeks with a 4 point assay buffer standard curve.
- Detection antibody stability on Edison Tips
  8 week stability test of detection antibody at working concentration in stabilizer stored at 4C, RT. Working concentration of conjugate tested against control at 0, 1, 2 ,4 ,8 ,12 and 24 weeks

6. Development Report

A concise development report saved on the T drive/Assay Systems/Assay Development reports/ to include

- Analyte background (Clinical range, special instructions for handling & storage)
- Commonly used ELISA kit (Hyperlink PDF Data sheet of Gold Standard)
- Antibody screen summary table
- Selected pair antibody information to include (Vendor, catalog #, current lot, specificity, epitope, stock concentration, working concentration)
- Link to or include the tip coating and assay protocol for running the assay on Edisons.
  - Specify analyte "in sample" concentrations and dilution ratio on Edisons

- Edison Protocol name and version
- Summary Graph and table for the assay optimization and validation experiments listed above.
- Calibration data and equation.

Links to experimental details of assays are saved in the following locations:

1. J drive
   J:\Experiment Log\An Experiment Log\Experimental log.xls

   This excel contains the index of all the individual assays and experiments. Details include Elog number, Assay developer information and Project name.

   The numeric Elog folders can be found at the location J:\Experiment Log\E0001 – E0999.

   These logs contain the specifics of each assay including reagents, assay optimization steps, raw data and completed analysis and reports.

2. T drive

   Summary documents for ELISA projects are detailed in the location below.
   T:\Assay_Systems\Assay Development Reports.

   This folder contains multiple sub folders that have been appropriately named. For e.g. the assays developed as part of the retail testing can be found in the folder named - Assays for Retail Cartridge 95th - 99th Percentile. The assays developed earlier a part of contracts for Pharmaceutical contracts are stored in the sub-folders – Completed assays and Current assays. These folders contain the details of the assay development process and the finalized conditions.

9/5/2013

Dear Elizabeth,

I would like to inform you that I have made the difficult decision of resigning from my position at Theranos. My last day will Wednesday, September 11th, 2013.

I would like to thank you for the opportunity to work at Theranos. I wish the company tremendous success in the area of healthcare. Please let me know how I can assist with the transition.

Sincerely,

Surekha Gangakhedkar