Parameters for Validation of cfDNA Biomarkers

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**Biomarker Assay Qualification and Validation**

**Qualification:** the evidentiary and statistical process linking a biomarker to biologic and clinical endpoints, i.e. “The Marker”

**Validation:** the process of assessing the performance characteristics of a given assay, i.e. “The Test”

- What’s the intended use?
  - Qualitative / Quantitative
  - This drives the design and performance characteristics

- Design and Develop Test
  - Verification – does the test perform as required?

- Generate Validation Data
  - Create plan, perform experiments, write report

- Implement Test
  - Performance monitoring / quality control
Categories of Biomarker Assays

5 categories of biomarker assays (as applied to protein biomarkers):

- **Quantitative**
  - **Definitive**: Uses calibrators to achieve absolute analyte quantitation.
  - **Relative**: Uses calibration standards, but purified reagents may not be available to achieve absolute quantitation, e.g. a cytokine assay.
  - **Quasi**: No calibration standards, but quantity can be expressed in terms of the test used, e.g. an antibody titre.

- **Qualitative**
  - **Ordinal**: No quantitation but using a relative scoring scale, e.g. staining intensity in immunohistochemistry.
  - **Nominal**: A ‘Yes – No’ readout.
**Categories of Biomarker Assays**

2 categories of biomarker assays (as applied to cfDNA biomarkers):

- **Quantitative**
  - **Definitive**
    - Uses calibrators to achieve absolute analyte quantitation
  - **Relative**
    - Uses calibration standards, but purified reagents may not be available to achieve absolute quantitation, e.g. a cytokine assay
  - **Quasi**
    - No calibration standards, but quantity can be expressed in terms of the test used, e.g. an antibody titre

- **Qualitative**
  - **Definitive**
    - Assays can unequivocally identify required marker / mutation
  - **Ordinal**
    - No quantitation but using a relative scoring scale, e.g. staining intensity in immunohistochemistry
  - **Nominal**
    - A ‘Yes – No’ readout
What Validation is Required?

- Biomarker assays need to be ‘fit for purpose’, i.e.:
  - Generate data of sufficient quality to meet the intended purpose of the clinical study, but
  - Validated to a stringency consistent with the intended use of the data
  - Defined by the ‘Biomarker Validation Decision Matrix’:

<table>
<thead>
<tr>
<th>Qualitative Purpose</th>
<th>Assay Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novel Discovery</td>
<td>Qualitative</td>
</tr>
<tr>
<td>PD Proof of Concept</td>
<td>Quantitative</td>
</tr>
<tr>
<td>Predictive Stratification</td>
<td></td>
</tr>
<tr>
<td>Prognostic Clinical Decision</td>
<td></td>
</tr>
<tr>
<td>Diagnostic Clinical Decision</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biomarker Purpose</th>
<th>Compliance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO QA</td>
<td>Full QMS</td>
</tr>
<tr>
<td>Accreditation</td>
<td></td>
</tr>
</tbody>
</table>

Increasing Stringency of Method Validation
# Validation Parameters for Qualitative and Quantitative Tests

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>True Positive Rate, i.e. True Positives / (True Positives + False Negatives)</td>
</tr>
<tr>
<td>Specificity</td>
<td>True Negative Rate, i.e. True Negatives / (True Negative + False Positives)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>Agreement between a test result and an accepted reference value (Quantitative)</td>
</tr>
<tr>
<td></td>
<td>“Sensitivity” plus “Specificity” (Qualitative)</td>
</tr>
<tr>
<td>Trueness</td>
<td>A measure of systematic error applied to Quantitative tests, expressed as % deviation from true value</td>
</tr>
<tr>
<td>Precision</td>
<td>A measure of random error between independent test results, usually expressed as SD or Coefficient of Variation. Applies to Quantitative tests</td>
</tr>
<tr>
<td>Limit of Detection</td>
<td>For Quantitative tests, the lowest amount of an analyte that can be statistically distinguished from background or a negative control. Can be applied to Qualitative tests, e.g. the lowest amount of DNA that can yield an informative mutation result.</td>
</tr>
<tr>
<td>Probability</td>
<td>Used in ongoing validation where the probability that a result is correct can be assigned</td>
</tr>
</tbody>
</table>

## Validation Parameters Applicable to Different Genetic Test Types

<table>
<thead>
<tr>
<th>Example</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Accuracy</th>
<th>Trueness</th>
<th>Precision</th>
<th>Limits of Detection</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quantitative:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Determination of % methylation</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Sizing a PCR product</td>
<td></td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Copy Number determination</td>
<td></td>
<td></td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td><strong>Qualitative:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation scanning for unknown mutations, e.g. by sequencing</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Genotyping for a specific mutation</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
</tbody>
</table>

++: Recommended parameter
+: Applicable parameter, less often used

Considerations for Test Development and Validation

– Sample Selection
  • Positive samples need to represent as broad a range of results as possible, to allow attainment of ‘diagnostic power’
    – May necessitate inter-lab collaboration!

– Sample Number
  • No specific guidelines, but in general is dictated by the ‘confidence level’ required (i.e. statistical power), but also sample availability, cost, time etc.....
    – Important to be able to generate data to assess specificity, selectivity, accuracy...
  • Equal numbers of positive and negative samples
  • Blinded analysis / randomisation to reduce systematic error
  • In any event, validation report needs to record all details and the basis for any decisions made
Considerations for Test Development and Validation (cont.)

– Reporting
  • Estimates of accuracy based on data analysis
    – Quantitative: Mean, SD, SE, **Upper / Lower Confidence Intervals**
    – Qualitative: Sensitivity / Specificity less meaningful as only relate to specific samples tested in validation…. Need confidence intervals associated with identifying unknown samples
  • Include context information, e.g. sample inclusion criteria, sample info, details of test method and any reference method, critical parameters measured, equipment details....

– Implementation
  • Quality Control
    – Monitoring and documentation of ongoing test performance
    – Identification of problems as they arise
  • Quality Assurance
    – Pre- and post-analytic as well as analytic
    – Include staff training, equipment monitoring, data and process documentation
Comparison of a multiple reference gene approach for plasma cfDNA quantification with single gene measurements. The 95 % confidence interval (grey area) associated with a normalised geometric average cfDNA quantity calculated on the basis of three reference genes (TERT, RPPH1, ERV3) and three independent qPCR experiments is compared with mean estimates for each of the above-mentioned reference genes and ALUJ for each donor.

cfDNA genomic copy numbers for 17 donors based on 7 reference assays:
ALUJ
TERT
RPPH1
ERV3
NAGK
GAPDH
VP (commercial assay based on undisclosed locus)
Plasma cfDNA levels at baseline correlates with survival in NSCLC patients

134 never smokers with advanced lung adenocarcinoma

Lee et al., Clin Cancer Res 17, 5179-5187 (2011)
Clinical validation of the detection of KRAS and BRAF mutations from circulating tumour DNA

Assessment of KRAS status is mandatory in patients with metastatic colorectal cancer (mCRC) before applying targeted therapy.

<table>
<thead>
<tr>
<th>Tumor-tissue analysis</th>
<th>KRAS</th>
<th>Mutant</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfDNA analysis</td>
<td>Mutant</td>
<td>36</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>3</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>39</td>
<td>56</td>
</tr>
<tr>
<td>BRAF</td>
<td>Mutant</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>All mutations</td>
<td>Mutant</td>
<td>41</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>44</td>
<td>51</td>
</tr>
</tbody>
</table>

96% concordance between tumour-derived and cfDNA


Limit of detection: 1 mutant sequence in 10,000 WT
Circulating Free DNA (cfDNA) as a Biomarker

- cfDNA can be released into the bloodstream via several mechanisms, and in several forms
  - Often fragmented: mean size ~100bp
  - Quantity can reflect tumour burden and rate of tumour proliferation
  - Can obtain cfDNA and CTCs from same CellSave tubes

Schwarzenbach et al., Nature Reviews Cancer 11, 426-437 (2011)