Flow Cytometry Biomarker Assays

Validation Criteria vs. Biology

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What is Flow Cytometry?

Measure the fluorescence properties of particles (normally cells) in a stream of fluid
What do we measure?

- Discovery
- Pre-clinical
- Clinical

+ Standard immunophenotyping
+ Cellular activation
+ Rare cell populations
+ Receptor Occupancy
What guidelines do we work to?

- No Flow specific guidelines
- Lee et al.
  - Not flow specific
- O’Hara et al. (2011)
  - Outlines standard Validation recommendation
## Standard Validation Design

<table>
<thead>
<tr>
<th>Test</th>
<th>Purpose</th>
<th>Design</th>
<th>Acceptance criteria</th>
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<tbody>
<tr>
<td><strong>Intra-assay</strong></td>
<td>Determine precision</td>
<td>n=3 to 6 animals n=3 replicates n=2 occasions</td>
<td>%CV ≤25%</td>
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<tr>
<td></td>
<td></td>
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<td>Rare cell events (&lt;5% parent population) %CV ≤30%</td>
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<tr>
<td><strong>Inter-analyst</strong></td>
<td>Determine variability between analysts</td>
<td>n=3 to 6 animals n=3 replicates n=2 analysts</td>
<td>%CV ≤25%; %Bias between analysts ≤25%</td>
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<td>Rare cell events (&lt;5% parent population) %CV ≤30%; %Bias between analysts ≤30%</td>
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<tr>
<td><strong>Inter-analyser</strong></td>
<td>Generate concordance and determine variability between instruments</td>
<td>n=3 to 6 animals n=3 replicates n=2 analysts</td>
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<td><strong>Inter-animal</strong></td>
<td>Generate a normal range of data</td>
<td>N=10 male, n=10 female animals</td>
<td>Range of values defined</td>
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<td><strong>Stability</strong></td>
<td>Determine fresh and processed stability</td>
<td>n=3 to 6 animals n=3 replicates Fresh T0hr Fresh and processed T24hr Fresh and processed T48hr</td>
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<td>Rare cell events (&lt;5% parent population) %CV ≤30%; %Bias from T0hr ≤30%</td>
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Simple immunophenotyping flow assay

- Basic immunophenotyping in blood samples
  - Simple sample preparation
  - Abundant cell populations
  - Extracellular markers
  - Population well defined

- T lymphocytes
- B lymphocytes
- NK cells
- Monocytes
- Neutrophils
Sample preparation

1. Aliquot samples
2. Incubate with Antibodies
3. Lyse and wash

3 hours

Offline-data analysis

2 to 3 hours
Complex data analysis

No Gate

WBC

Lymphocytes

B cell

T cell

Neutrophil

Monocyte

NK Cells

CAL beads
<table>
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<tr>
<th>Animal ID</th>
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<th>WBC % Cells/µL</th>
<th>Lymphocytes % Cells/µL</th>
<th>T cells % Cells/µL</th>
<th>B cells % Cells/µL</th>
<th>NK cells % Cells/µL</th>
<th>Neutrophils % Cells/µL</th>
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<td>2.5 30.0</td>
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<td>1.8 4.0</td>
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</table>

Large data-sets
Definition of a clarifying criteria

+ What happens when not all animal/donors pass a given test?
  + Has the test has passed or failed?

+ Defined a new clarifying criteria that \( \frac{2}{3} \) of tests must pass to be considered to have passed the acceptance criteria

+ Increase animal number to improve confidence in the result
  + n=6 animals
Summary so far and more complex assays…

+ Criteria generally appropriate for simple assay however need a few more decision making tools:
  + 2/3 samples passed at acceptance criteria

+ Simple!

+ But… complex assays are more challenging
  + Complex sample preparation
  + Rare cells (CD34+ stem cells in blood)
  + Intracellular markers (Ki67, proliferation)
  + Intracellular markers that are rare (FoxP3, regulatory T cells)
  + Intracellular markers that are only expressed upon stimulation (pSTAT, activation)
  + Intracellular markers that are rare and only expressed upon stimulation…
Rat Th17 and Treg assessment

+ Remit:
  + Measure proportion of Th17 and regulatory T cells (Treg) in rat PBMC (peripheral blood mononuclear cell) preparations

+ Challenges
  + PBMC preparation challenging with low volumes of blood
  + Tregs
    + rare cell population
    + uses an intracellular marker (FoxP3)
  + Th17
    + rare cells population
    + uses IL-17 as a marker, which is only present upon stimulation and suppression of excretion intracellularly…

+ Not so simple!
Sample preparation

1. Prepare PBMCs
2. Aliquot samples
3. Stimulate 4 hours
4. Incubate with antibodies
5. Lyse, wash, fix and permeabilise

8 hours, Day 1

Offline-data analysis

2. Incubate overnight

2 hours, Day 2

5. Incubate with antibodies
6. Wash

5 hours, Day 2

13
Data analysis for complex assay

Lymphocytes

T lymphocytes

CD4+ T Lymphocytes

Unstimulated

Stimulated

CD3 FITC

CD4 PE-Cy5

IL-17a PE

FoxP3 eFluor660

FoxP3 eFluor660
Choose the relevant populations to assess

+ **Treg values assessed from Unstimulated**
  + Population may have changed in stimulated samples, therefore unstimulated sample is as close as possible to original sample

+ **Th17 values assessed from Stimulated**
  + IL-17 only produced in stimulated cells, therefore cannot be detected in the unstimulated sample
Treg

Passed all criteria except stability at \( \leq 30\% \)

Th17

Generally failed… Why?
## Th17 Assessment

### Intra-assay 1

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<th>Animal ID</th>
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<th>Th17</th>
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Why did Th17 fail?

+ Rare cell event
+ Also subject to:
  + Variability in sample preparation
  + Robust stimulation
  + Acceptable permeabilisation to allow antibody to access the antigen inside the cell
  + Complex data analysis, with gating set on the unstimulated sample

+ Are we assessing this parameter appropriately?
Used ‘cut-point’ style approach

+ Defined a ‘cut-point’ from inter-animal assessment
  + Cut-point defined in unstimulated samples
    + Any value above this has been stimulated appropriately and can be classed as a ‘positive’
  
+ Range defined in stimulated samples
  + Any deviation from this value on study can be considered a change from the normal range

+ Need higher animal number
  + As this study wasn’t designed for a cut-point approach insufficient animals to define a tolerance

+ Allows us to define a positive sample and where a change from ‘normal’ has been observed in sample analysis
Simple assay variation

1. Aliquot samples
2. Incubate with Antibodies
3. Lyse and wash
4. Offline-data analysis
5. 2 to 3 hours
Sources of variation for simple assay

- Biological
  - Aliquots
    - Instrumental
      - Data analysis
  - Analyst
    - Instrumental
      - Data analysis
  - Analyser
    - Instrumental
      - Data analysis
  - Antibodies
    - Instrumental
      - Data analysis
Sources of variability

1. Prepare PBMCs
2. Aliquot samples
3. Stimulate 4 hours
4. Incubate with antibodies
5. Lyse, wash, and permeabilise

8 hours

6. Incubate overnight
7. Incubate with antibodies
8. Wash
9. Offline data analysis

2 hours

5 hours
Sources of variation for simple assay
In complex analyses is there so much potential variation that the traditional replicate analysis approach not sufficient?

Understanding the biology of the system and the sources of variation is key
  + We should apply this knowledge to our validation design

What are important factors to consider for flow analyses?
  + Biological variability and sample processing
  + Detection of rare cell events
  + Number of cells counted through the instrument, e.g. LLOQ
  + Gating and LOD (limit of detection) from FMO (fluorescence minus one) samples or unstimulated samples
Acknowledgements

+ The Biomarker, Bioanalysis and Clinical Sciences Team:
  + Adrian Freeman
  + Asha Lad
  + Karen Cadwallader
  + Tom Scott
  + Yulia Tingle
  + Usha Agarwala
  + Yvonne Whitehead
  + Emma Keen
  + Gareth Thomas, Statistics

+ Caroline Gennell, GSK
The BBC Team