Multiplex Real Time PCR: The Good, The Bad And The Downright Ugly

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What is multiplex PCR?

• Multiplex real time PCR
  – Detection of more than one target in a single PCR reaction/tube
    • A multi-pathogen test
    • Any assay that includes an IC
    • A diagnostic/typing assay

• Has many advantages and pitfalls.
The Good: Original testing protocol

- Originally our service was based on **pathogen** testing
  - Large number of individual assays
  - Complicated/inconsistent coding
  - Prolonged TRT
  - Users “drip fed” results
  - More costly
## Syndromic testing: reducing the number of tests

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of tests</th>
<th>Pathogens tested for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory sample</td>
<td>5</td>
<td>influenza A, B, C; RSV A + B; adenovirus; rhinovirus (A,B and C), Enterovirus; PF1, 2, 3, 4; coronavirus NL63, 229e, OC43; HKU1, HuMPV A + B; <em>M. pneumoniae</em></td>
</tr>
<tr>
<td>Respiratory sample from IC</td>
<td>6</td>
<td>Above test AND Multiplex for: CMV, PCP and IC</td>
</tr>
<tr>
<td>Vesicle Fluid</td>
<td>1</td>
<td>HSV1/2/VZV</td>
</tr>
<tr>
<td>Genital</td>
<td>1</td>
<td>HSV-1/2/Syph</td>
</tr>
<tr>
<td>Eye swab</td>
<td>1</td>
<td>Adeno HSV-1/2 Chlamydia VZV</td>
</tr>
<tr>
<td>Blood (transplant)</td>
<td>1</td>
<td>Adeno/CMV/EBV</td>
</tr>
<tr>
<td>CSF</td>
<td>2</td>
<td>Entero/Parecho HSV-1/2/VZV</td>
</tr>
</tbody>
</table>

*Note: CMV = Cytomegalovirus, PCP = Pneumocystis Pneumonia, IC = Influenza A, B, C, RSV = Respiratory Syncytial Virus, HSV = Herpes Simplex Virus, VZV = Varicella Zoster Virus, Adeno = Adenovirus, Chlamydia = Chlamydia, EBV = Epstein-Barr Virus, Parecho = Parechovirus,*
Test costs are reduced

<table>
<thead>
<tr>
<th>Sample</th>
<th>Annual total (based on 2006 data)</th>
<th>Number of tests carried out prior to service changes</th>
<th>Number of tests carried out (post multiplex)</th>
<th>Total savings in terms of test costs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>~2,000</td>
<td>8,000</td>
<td>2,000</td>
<td>75%</td>
</tr>
<tr>
<td>CSF</td>
<td>~1,800</td>
<td>12,600</td>
<td>7,200</td>
<td>43%</td>
</tr>
<tr>
<td>Vesicle fluid</td>
<td>~2,000</td>
<td>4,000</td>
<td>2,000</td>
<td>50%</td>
</tr>
<tr>
<td>Transplant</td>
<td>~3,000</td>
<td>9,000</td>
<td>3,000</td>
<td>66%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10,800</strong></td>
<td><strong>35,600</strong></td>
<td><strong>16,200</strong></td>
<td><strong>55%</strong></td>
</tr>
</tbody>
</table>
Coding become less specialised

<table>
<thead>
<tr>
<th>SPECIMEN TYPE</th>
<th>STUDY CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invest</td>
<td>result</td>
</tr>
<tr>
<td>DIF</td>
<td>PCRVF</td>
</tr>
<tr>
<td>DIF1-5</td>
<td>PCRGEN</td>
</tr>
<tr>
<td>DIF7</td>
<td>PCREYE</td>
</tr>
<tr>
<td>PCRRUT</td>
<td>PCRCSF</td>
</tr>
<tr>
<td>BAL</td>
<td>PCRNoro</td>
</tr>
<tr>
<td>PMR</td>
<td>PCRGAS</td>
</tr>
<tr>
<td>PMH</td>
<td>PMF</td>
</tr>
<tr>
<td>PCRSTD: split sample at</td>
<td></td>
</tr>
<tr>
<td>reception</td>
<td></td>
</tr>
<tr>
<td>Preliminary Results</td>
<td></td>
</tr>
<tr>
<td>Coded By:</td>
<td>Checked By:</td>
</tr>
<tr>
<td>Re-coded By:</td>
<td></td>
</tr>
</tbody>
</table>
Reduction in TRT achieved when using multiplex test (in days) *

- Eye test
- Tx screen

* Reduction in TRT (in days) when using multiplex tests compared to traditional methods.
Simultaneous Detection and Quantitation of Cytomegalovirus, Epstein-Barr Virus, and Adenovirus by Use of Real-Time PCR and Pooled Standards

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Quantitative real-time PCR has become the most widely used preemptive approach for managing cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus infections in immunosuppressed patients. These three assays are normally available as separate tests, each using five quantitation standards that are tested in duplicate. We have developed an adenovirus–CMV–EBV triplex assay that uses one set of five pooled quantitation standards, tested singly rather than in duplicate. This test demonstrated a sensitivity and an accuracy of quantitation equivalent to those of our previous single tests and was shown to be able to detect mixed infections with no loss in sensitivity. This assay is now in routine use in our laboratory and has considerably simplified the workflow of the laboratory, with a resultant improvement in sample turnaround time and significantly reduced costs.
Summary

• Advantages of multiplex real time PCR:
  – Less tests
    • Favours higher throughput
    • Favours automation
  – Less costly
    • Favours adding tests
  – Easier coding/reporting
  – Reduced TRT
The Bad.........
Design issues that limit the number of targets multiplexed.

• Design issues
  – not all combinations of tests will work together without compromising test performance.
    • influenced by PCR mastermix
    • ?affect retrospective incorporation of IC
  – multiplexing is still limited to 4 targets/reporter dyes
    • Limited number of detector channels on commonly used PCR platforms
Different lots of primers/probe may not be of consistent quality

- Different lots of primers/probe may not be of consistent quality
  - Optimal concentrations of primers/probes differ significantly between lots
  - Between suppliers and from the same supplier.
New lots can be less sensitive than previous lots. Re-optimisation may rectify the issue but equally often will not.
New lots can be less specific

Reducing CMV F primer concentration eliminates cross-reaction
Test Competition

• Competition for PCR mastermix between two or more different components of an multiplex assay.
  – the IC and positive target
  – Samples containing >1 target.
  – Assays that detect and type simultaneously

• The stronger target can be preferentially amplified to the detriment of the weaker target.

• Has numerous effects on test performance
Can reduce sensitivity/flat curves

These are the traces for two tests of the same dilution series of PF3. All the dilutions also contain an IC at a Ct of ~30. The dilution series on the left was tested using a single PF3 test whereas the dilution series on the right was tested using a PF3/IC duplex.
Competition can result in false negative results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Single testing</th>
<th>Multiplex assay (using non multiplex kit)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adeno</td>
<td>CMV</td>
</tr>
<tr>
<td>A</td>
<td>31.04</td>
<td>31.34</td>
</tr>
<tr>
<td>B</td>
<td>11.46</td>
<td>28.86</td>
</tr>
<tr>
<td>C</td>
<td>12.12</td>
<td>31.50</td>
</tr>
</tbody>
</table>
Can reduce linearity/accuracy

This slide shows a BK dilution series (10 fold). All the dilutions also contain an IC at a Ct of ~29. The dilution series on the left was tested using a single BK test whereas the dilution series on the right was tested using a BK/IC multiplex test.
Can affect interpretation of the IC

These are the results of the IC component of a dilution series of HCV (ct 23-37). Each should be positive with a Ct ~30. The stronger the HCV the more affected the IC result.

Note: weak HCV results (50IU/ml) were also out competed by the IC.
Competition can be overcome.

- Primer limitation experiments
  - Useful for incorporation of an IC
  - Increases the complexity
  - Balance between test sensitivity and competition
  - Doesn’t always eliminate the problem entirely.

- Use an alternative PCR kit.

- Use a multiplex PCR kit
  - Specially designed to amplify >1 target simultaneously.
  - RNA and DNA kits available

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</tr>
<tr>
<td>C</td>
<td>12.12</td>
<td>31.50</td>
<td>30.42</td>
</tr>
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</table>
Multiplex Kits can lead to false positives
Test competition

Is important because:

• Number of clinical samples likely to have >1 pathogen
• All assays aim to incorporate an IC
• Varies by kit and primer/probe optimisation
  – problem likely to vary lab to lab
• Not assessed by EQA
• **May affect the utility of commercial pooled controls/standards**
Summary

• Multiplex real time PCR has many advantages.

• Important to be aware of pitfalls associated with these assays

• The pitfalls described here:
  – Can affect the performance of a test both within a lab and between laboratories.
    • Can affect the retrospective incorporation of IC component.
Acknowledgements

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