Pitfalls of PCR diagnosis of viral infections

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**PCR & viral infections**

PCR technology has revolutionised diagnosis. However, like any technology, there can be some problems.

- Assays needs to be carefully designed & evaluated.

- So what makes a **good** PCR assay?
  
  - best answered by showing examples of potential problems….
Sequence variation
Example:

False-negative results caused by sequence variation
- probe
- primer
Eg. Parainfluenza type 3

- Used two different TaqMan real-time assays for the detection of parainfluenza 3 in NPA specimens from local Brisbane population

- 33 samples positive by both assays

- HOWEVER:
  1 sample positive by only one assay
Eg. Parainfluenza type 3

Gel electrophoresis....
Eg. Parainfluenza type 3

Amplification product was present for assay 2

TM Probe 5’ TCAATCATGCAGTGCTCTCAACAGAGCTTG 3’
Specimen TCAAT TATGCGATC CCAACAGAGCTTA

POS NEG specimen
### Eg. Other viruses...

<table>
<thead>
<tr>
<th>Organism</th>
<th>Assay(s) affected</th>
<th>Assay target</th>
<th>Basis of false-negative results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytomegalovirus</td>
<td>in-house PCR method</td>
<td>glycoprotein B gene</td>
<td>Up to three mismatches in probe target</td>
<td>Nye et al. 2005</td>
</tr>
<tr>
<td>HIV Type 1</td>
<td>Amplicor HIV-1 (Roche)</td>
<td>gag gene</td>
<td>Up to six mismatches in primer targets</td>
<td>Barlow et al. 1997</td>
</tr>
<tr>
<td>Parvovirus B19</td>
<td>LightCycler parvovirus B19 Quantification kit (Roche), RealArt Parvo B19 PCR Kit (Artus)</td>
<td>NS gene</td>
<td>Mismatches in primer targets</td>
<td>Cohen et al. 2006</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>in-house PCR method</td>
<td>N gene</td>
<td>Up to six mismatches in the probe target and up to four in the primer targets</td>
<td>Hughes et al. 2004</td>
</tr>
</tbody>
</table>

Example:

*Reduced fluorescent signal caused by sequence variation*
Reduced fluorescent signal

Eg. Minor groove binder (MGB) TaqMan probe-based assay for RSV.

RSV MGB Probe  5′ TCAATACCAGCTTATAGAAC 3′
Specimen 1       TCAATACCAGCTTTATAGAAC
Specimen 2       TC\textcolor{red}{T}ATACCAGCTTATAGAAC
Specimen 3       TCAATACCAGCTTAC\textcolor{red}{G}AAC
Example:

Error in quantitative PCR caused by sequence variation
Error in qPCR results

Example: BK polyomavirus

- A specimen was tested by two BKV quantitative PCR assays and very different results were obtained:

  Assay 1: 1,000,000 copies per mL
  Assay 2: 1,000 copies per mL
Error in qPCR results

Assay 2 primer 5’ GTAAAAGACTCTCTGTAAAGACTCC 3’
Specimen GTAAAAGACTCTCTGTAAAGACTCCG
Error in qPCR results

- Mismatches in primer targets can introduce considerable error (numerous logs).

- Overall impact is dependent on the particular assay:
  - position of mismatches
  - nucleotide composition of the primers
  - annealing temperature
  - reaction mix composition

MUST validate a PCR for quantitative use (cf qualitative use)
  - Particularly for viruses showing much variation
    eg. RNA viruses etc.

*Wiley DM, Sloots TP.*
Sequence variation in primer targets affects the accuracy of viral quantitative PCR.
Example:

Problems with melting curve analysis (using hybridisation probes) caused by sequence variation
Melting curve analysis typing failure

Eg. HSV

- Hybridisation-probe based method (Espy et al.)

- Most HSV strains are able to be typed as type 1 or 2 by melting curve analysis.

- However, some strains provide melting peaks that are not characteristic of either HSV type 1 or 2.
Eg. HSV typing
Melting curve analysis typing failure

Eg. HSV

- Untypeable strain was a HSV type 2 strain containing 3 mismatches with probe 2. (There should be no mismatches with HSV type 2).

<table>
<thead>
<tr>
<th>Probe 1</th>
<th>Probe 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ GTACATCGGCCTCATCTGCGGGGGGCAAG TGCTCATCAAGGGGCGTGGATCTGGTGC 3’</td>
<td>Spec GTACATCGGCCTCATCTGCGGGGGGCAAGATGCTCATTAAGGGCGTGCGACCTGGTGCGC</td>
</tr>
</tbody>
</table>

- These 3 mismatches lowered the melting temperature of this HSV type 2 strain by 14 °C, preventing the determination of HSV type.
Melting curve analysis typing failure

Hybridisation probes – typing issues:

- Very useful technique, BUT...
- Uses a large target sequence (up to 50 bases) to detect a few SNPs
- Therefore very susceptible to further variation.
General ways to combat / deal with sequence variation...
The Use of Multi-Target Assays;

- **Two sequence targets.**
  Rationale: by targeting two different regions there is less chance of variation occurring in both sequences, reducing the potential for false-negative results.

**Routine use –**

- Commercial dual-PCR target HIV-1 Test (Roche)
The Use of Multi-Target Assays;

• **Two sequence targets.**
  Rationale: by targeting two different regions there is less chance of variation occurring in both sequences, reducing the potential for false-negative results.

Research?
• newly described organisms.
   Eg. hMPV
   Our original single target hMPV PCR (2002) was designed using limited sequence data.
   We missed a second lineage of hMPV in our population

• **We now use at least two different assays targeting different gene targets when investigating novel or newly described organisms.**
Enhanced QA?

• Batch retesting of samples (pooled?) by an alternative method?

Eg.
• We recently ran patients with suspected resp virus infection, but negative by our PCRs, through the Abbott Ibis Resp viral panel:
  ◆ Identified an Adenovirus variant.
  ◆ Was negative by our Adenovirus PCR.
  ◆ Now redeveloping adenovirus PCR.
Staying up-to-date with sequence information.

- Regularly checking sequence databases (eg. Genbank) for potential problems with primer or probe targets.

- Eg. Influenza A: GISAID.
Problems with multiplex PCR assays
**Multiplex assays – the basics:**

- Two or more PCR reactions (targeting different templates) are incorporated into the one reaction mix.

**BENEFITS –**

- Reduced cost

- Reduced hands-on-time
  - fewer reaction mixes to make, store, QC etc.
  - Fewer reactions per sample to load

- Higher throughput
  - saves valuable space on real-time PCR instrumentation

**THINGS TO WATCH OUT FOR –**

- Reduced sensitivity
  - Caused by **competitive inhibition**: the earlier amplification of one reaction inhibits the amplification of a second reaction.
Example:

Competitive inhibition caused by non-specific primer interactions (primer dimer)…
Primer dimer:
• Is a non-specific product caused by the primers interacting/amplifying with each other.
• Can cause problems in multiplex PCR assays as there may be many different primers in the same reaction mix.
Assay evaluation/optimisation: Here a multiplex PCR was compared with a single PCR. Note that 10-fold dilutions of parainfluenza 1 RNA were used.

Para 1 singleplex

Para 123 Triplex

Looks OK??
Assay evaluation/optimisation: Here a multiplex PCR was compared with a single PCR. Note that 10-fold dilutions of parainfluenza 1 RNA were used.
Example:

Extreme competitive inhibition caused by competition between specific primer reactions -

“consensus” primer sequences.
Extreme competitive inhibition:

Eg. Detection of HSV types 1 and 2

• “consensus” primers and probes - some assays use the same primers and probes to amplify both HSV type 1 and type 2, and then distinguish the viruses by melting curve analysis. Eg. LightCycler hybridisation-probe based methods.

• “type-specific” primers and probes – other assays use separate primer and probe sequences for each HSV type. Eg. TaqMan-based methods.

The above can have implications where there are mixed infections...
Extreme competitive inhibition:
Eg. Detection of HSV types 1 and 2: “consensus” vs “type-specific”

Consensus primers and probes: *Hybridisation probe assay*

Type-specific primers and probes: *Duplex TaqMan probe assay*
Eg. Detection of HSV types 1 and 2

<table>
<thead>
<tr>
<th>Dilutions:</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tbody>
<tr>
<td>Copies of HSV type 1</td>
<td>10E7</td>
<td>10E6</td>
<td>10E5</td>
<td>10E4</td>
<td>10E3</td>
<td>10E2</td>
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<td>Copies of HSV type 2</td>
<td>10E4</td>
<td>10E4</td>
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Results:

**Hybridisation probe assay**

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<th>HSV type 2</th>
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**Duplex TaqMan probe assay**

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**Consensus primer sequences:**

**Note:**

- Where a primer pair can amplify 2 different targets, and both targets are present in a specimen, the PCR will favour the target at greatest concentration.

- Generally will only detect both targets when their relative difference in concentration does not exceed one log.

*Whiley DM, Sloots TP.*

*Melting curve analysis using hybridization probes: limitations in microbial molecular diagnostics.*

**Consensus primer sequences:**

**Note:**

- If the detection of a particular viral type carries clinical importance then **type-specific primers and probes** should be used in preference to consensus oligonucleotide sequences.
  - Eg. Consensus primers used to amplify JC and BK
    - Presence of JC in urine can prevent detection of BK
    - Important to detect BK reactivation in transplant patients.

- May be best to use **individual assays for some viruses**: ie. not multiplex.
Poor quality reagents?
Reagent quality can vary between batches.
- highlights the importance of good quality control:
  - Can affect:
    - Extraction reagents.
    - Reaction mix
    - Primers
    - Probes
    - etc…. 
Reagent quality can vary between batches.
- highlights the importance of good quality control:

Reaction mix problems?

Old mix

New mix
Reagent quality can vary between batches.
- highlights the importance of good quality control:
So what makes a **good** PCR assay?

- **Where assay design, evaluation & routine use takes into account:**
  - The intended purpose of the assay
  - Up-to-date sequence information
  - The use of good quality control (i.e., optimal reagents)
  - Any other relevant information? (may be organism-specific)