Sample number and denaturation time are crucial for the accuracy of capillary based LightCyclers.

Capillary-based LightCyclers

- Cooling and heating of samples by airflow
- Reactions performed in glass capillaries
- Capillaries placed on the perimeter of a carousel
- Carousel rotates >>> increased precision
Capillary-based LightCyclers

- Use in mutation screening: search for deletions and duplications
- $C_t$ difference of 1 between calibrator and sample: deletion
- $C_t$ difference of 0.5 between calibrator and sample: duplication

There must be a problem...

- Quantitative analysis of CpG-islands: denaturation temperature increased to 98°C
- Dilution series for SNRPN-locus: there must be a problem with this assay
... with the LightCycler

• Technical replicates show: it’s the instrument.

95°C : similar effect

• Denaturation 95°C: max. $C_t$ difference > 0.5
  >>> duplication in diagnostic set-up
Other instrument

- Other instrument at 95°C: max. $C_t$ difference > 0.9
  >>> deletion in a diagnostic set-up

CFTR primers: no such effect

- Use of CFTR primers >>> no such effect

![Graph showing cycle over threshold against carousel position for SNRPN and CFTR primers.](image-url)
Explanation: Wilhelm et al 2000

- Temperature inhomogeneities in thermal chamber:
  if template has high $T_m$, there is an effect on $C_t$
  
  > At hot positions in thermal chamber
  > more denatured gDNA molecules
  > increased accessibility for primers
to gDNA
  > lower $C_t$

>>> Explains why effect is not
observed in CFTR assay:
low melting temperature
of template compared to
SNRPN assay

Filling carousel: does it help?

- Are temperature inhomogeneities due to airflow
disturbances caused by missing capillaries?

  > positions 27-32 filled
  with capillaries
Filling carousel: does it help?

- Are temperature inhomogenities due to airflow disturbances caused by missing capillaries?

> positions 27-32 filled with capillaries
> effect disappears

- other LC instrument:
  effect does not disappear with filled carousel
> T-inhomogeneity inherent in chamber
Carousel rotation: does it help?

- Carousel rotates with 1/5 rotation per second

>>> thermal chamber inhomogeneities should be leveled off – but denaturation time is 0 seconds

> Carousel filled, denaturation time increased to 5 seconds
> effect disappears also on 2nd instrument

Summary LightCycler

<table>
<thead>
<tr>
<th>Method</th>
<th>$C_t$ average</th>
<th>$\Delta C_t$ max</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carousel not filled, 0 seconds denaturation</td>
<td>24.48</td>
<td>$\geq 0.94$</td>
<td>$\geq 1.28%$</td>
</tr>
<tr>
<td>Carousel filled, 5 seconds denaturation</td>
<td>23.67</td>
<td>$\leq 0.11$</td>
<td>$\leq 0.13%$</td>
</tr>
</tbody>
</table>

>>> To obtain the precision necessary for reliable deletion/duplication screenings, all positions on the LightCycler carousel have to be occupied with capillaries, and the denaturation time has to be set to 5 seconds
Pierce et al 2004: TaqMan assay for mRNA quantification, performed on ABI 7700

>>> Similar effects also on plate-based platforms, and even with cDNA

>>> Always think about positional effects!

Conclusions

• Capillary-based LightCyclers:
  - Set denaturation time to 5 seconds
  - Completely fill the carousel
  >>> Instrument gets precise in all assays

• All real-time PCR instruments:
  - Instruments precision should also be assessed with “difficult” primer pairs and templates
  - Assessing and improving instruments precision should be the first step when establishing qPCR assays
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