The Clamped-Probe-Assay: Detection & quantification of minor/minimal variants.

Olfert Landt  qPCR symposium march 2005, Leipzig
qPCR means Real-Time PCR
Real-Time PCR is based on Fluorescence
Real-Time PCR is based on Fluorescence

- λ: wavelength (nm)
- ε: extinction coefficient
- q: quantum yield

Absorbtion Maximum
Emission Maximum
Stoke's Shift
Quench
FRET (Fluorescence Resonance Energy Transfer)
Photobleaching
Background fluorescence
Lifetime

Fluorescence is less sensitive when compared to radioactivity or enzyme-linked reactions.
quantitative PCR itself is not very exciting

Real-Time PCR publications

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As a producer of fluorescent probes we are more interested in new (PCR) technologies.
Molecular Concepts: Hybridization Probes

Principle: adjacent hybridisation and FRET

**Excitation**
- Fluorescein (donor)
- LC RED640/705 (acceptor)
- Phosphate

**Non-detected emission**

**Detected emission**

Annealing

AMPICLON

1-5nt
How quantification works

The probes monitor the actual amount of PCR-product

The slope of the log $C$ (of the target concentration) is proportional to the cycle number. The amount of an unknown sample can easily be read out from this standard curve.

Slope = -3.640
Intercept = 39.35
Error = 0.0157
$r = -1.00$
How genotyping works

- Single Nucleotide Polymorphism (SNP)
- each mismatch destabilizes hybridisation strength
- the melting temperature is lowered for mismatched probes
- Use a pair of long (high Tm) anchor and short sensor probe
Example: hemachromatosis (HFE)

- **Homozygous**
- **Mismatches**
- **Heterozygous**

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Example: Identification of bacteria

- Gram positive
- Brucella
- Gram negative
Problem: Low-abundance (geno)types

G A
1:1

G G
5:1

G
100:1

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Example: k-ras Codon 12

- Several mutations
- Ratio: 1:10,000

Melting on artificial targets

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PNA/LNA-mediated PCR-clamping

WT

MU

DNA

PNA

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PNA/LNA-mediated PCR-clamping

WT

10,000

MU

1

PNA

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Molecular concepts: Clamped-Probe-Assay

Possible applications:

- detection of minor variants (e.g. k-ras codon 12,13)
- minimal residual diseases (MRD)
- developing resistances (STI-571 in abl exon 6 in CML, developing lamivudine resistance in HBV, ...)

Melting temperature

AMPLICON

- $-dF/dT$

wt-specific PNA

no signal

$50 \ 60 \ 70 \ 80 \ 90$

$50 \ 60 \ 70 \ 80 \ 90$

- $dF/dT$

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Clamped-Probe-Assay: k-ras Codon 12

k-ras F

ATAAGGCTGTGAAAATGACTGAAATATAAACTTGTGTAGTTGGAGATTGAGCGGTGGCCCTAGGCAAGAGTGCCTTGACGATACAGCTAATTTCAAGACTACTTTTGT
TATTCCGGGACGACTTTTTACTGACTTTATATTGAAACACACCATCAACCTCCACCCAGCCTCGTCCTCCACCGGACTGCTATGTTCATTTAGTTAAAGAC

K-ras Exon 1

ATG

K-ras Intron 1

GACGAATATGATCCACAAATAGAGGTAAATCTTTTTATATCAGATTTAATGCTGTACGCGACCTTTTCTTCTTACAGATAAAAGGTTTCTCTGACCATTITCA
CTGCTTATTACTAGGTGGTTATCTCCATTTGAGAAACAAATTATACGTATAATGACCACGTCCCTGTTAAAGAAAACATAGTCTATTTCCAAAGAGACTGGTAAAGT

PNA-15/16

k-ras Anchor

12Cys Sensor (LC705)

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Melting curves from different patients

Melting curve (fluorescence vs. temperature)

- Mutant 12Cys
- Wild type (clamped)

- 68°C

Melting curve (dFl/dT vs. temperature)

- Different clinical samples containing k-ras Codon 12 mutations
- 65°C
- 61°C

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Dilution row of mutant in wt DNA

Melting curve (fluorescence vs. temperature)

Amplification plot (normalized)

Melting curve (dFl/dT vs. temperature)
Clamping probes consisting of Locked-Nucleic Acid (LNA) work even well.

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Chemistry of the clamping probe</th>
<th>Concentration of probe still suppressing the 12 Gly-variant</th>
<th>Concentration of probe suppressing the 12 Cys-variant (1 mismatch)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PNA 15mer 12Gly</td>
<td>2,8 µM*/3 µM§</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>PNA 17mer 12Gly</td>
<td>1,0 µM</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>LNA 17mer 12Gly</td>
<td>0,1 µM</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5'-NH LNA 17mer</td>
<td>0,05 µM</td>
<td>0,2 µM</td>
</tr>
<tr>
<td>5</td>
<td>5'-MB LNA 17mer</td>
<td>0,02 µM</td>
<td>0,1 µM</td>
</tr>
<tr>
<td>6</td>
<td>3'-NH LNA 17mer</td>
<td>0,1 µM</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3'-MB LNA 17mer</td>
<td>0,05 µM</td>
<td></td>
</tr>
</tbody>
</table>

A 3'-terminal attached MB dye boosts the suppression significantly (the working concentration is 20-fold lower).
Future targets for the CPA assay: CML

The M315T mutation in ABL exon 6 and mutations at Y253 and E255 in ABL exon 4 are responsible for the resistance of bcrABL-clones in CML.


Future targets for the CPA assay: CML

Serial dilutions of mutant in wildtype, wildtype control and patient sample.

Patient history monitored with the CPA assay.

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2005 : TIB moves into our new building

TIB MOLBIOL (oligo synthesis)
GenExpress (cloning services)
Chipron (low-cost/low-density array)
Chemicell (magnetic beads)
emp biotech (fluorescent reagents)
New technologies ... for example chips
Precise quantification is a function of standards.