Finding the needle in the haystack
LNA bases enhance SNP detection dramatically

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Weihenstephan, September 2005
Real-Time PCR is based on Fluorescence

Fluorescence is less sensitive when compared to radioactivity or enzyme-linked reactions.

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

Absorption Maximum
Emission Maximum
Stoke's Shift
Quench
FRET (Fluorescence Resonance Energy Transfer)
Photobleaching
Background fluorescence
Lifetime
Real-Time-PCR Instruments

- **LightCycler 480**: Roche Diagnostics
- **LightCycler**: Roche Diagnostics
- **LightTyper**: Roche Diagnostics
- **SmartCycler**: Cepheid
- **Opticon**: MJ Research
- **iCycler**: BioRad
- **MiniOpticon**: MJ Research-BioRad
- **RotorGene**: Corbett Research
- **Mx3000/4000**: Stratagene
- **SDS7000**: Applied Biosystems
- **SDS7700**: Applied Biosystems
- **Superconvector**: AlphaHelix
- **7900HT**: Applied Biosystems

**September 2005**
As a producer of fluorescent probes we are interested in new PCR detection technologies.
Molecular Concepts: Hybridization Probes

Principle: adjacent hybridisation and FRET

Fluorescein (donor)  LC RED640/705 (acceptor)  Phosphate

Annealing

EXCITATION  NON DETECTED EMISSION  EXCITATION  DETECTED EMISSION

AMPLICON

1-5nt

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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 a unknown sample can easily read out from this standard curve.

- Slope = -3.640
- Intercept = 39.35
- Error = 0.0157
- $r = -1.00$

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Real-Time qPCR itself is not very exciting

Real-Time PCR publications


Number of publications:
- 0 in 1994
- 100 in 1995
- 150 in 1996
- 200 in 1997
- 250 in 1998
- 300 in 1999
- 600 in 2000
- 1000 in 2001
- 1500 in 2002
• 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**
- **Heterozygous**
- **Mismatch**
- **Match**
Example: Identification of bacteria

- **Gram positive**
- **Brucella**
- **Gram negative**
q(Geno-)Typing

Detection of sequence variations (mutations) and how to find minimal amounts of variants, e.g. contaminations, minimal residual diseases, or growing resistant populations, varying in just one base.
Problem: Low-abundance (geno)types

- 1:1 ratio:
  - G:A
  - Height of G is twice that of A

- 5:1 ratio:
  - G:A
  - Height of G is five times that of A

- 100:1 ratio:
  - G:A
  - Height of G is 100 times that of A
Example I: k-ras Codon Gly12

- Several mutations
- Ratio: 1:10,000

Melting on artificial targets
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, ...)
Example I: k-ras Codon 12

[Image of a diagram illustrating the k-ras gene at Codon 12, showing the positions of exons and introns.]
Melting curves from different patients

Melting curve (fluorescence vs. temperature)

- Mutant 12Cys
- Wild type (clamped)

Melting curve (dFl/dT vs. temperature)

- Different clinical samples containing k-ras Codon 12 mutations
  - 68°C
  - 65°C
  - 61°C

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Dilution row of mutant in wt DNA


Clamping probes consisting of Locked-Nucleic Acid (LNA) work even well.

A 3'-terminal attached MB dye boosts the suppression significantly (working concentration is 20-fold lower).
Example II: JAK2 mRNA or gen. V617F

**Graph:**
- X-axis: Temperature (°C)
- Y-axis: Fluorescence (arb. units)

**Legend:**
- Val
- Phe
- Val+LNA
- Phe+LNA

**Sequence:**
- JAK2 F
- V617F F

**毂内含量变化图示**

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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.


Example III: Leukemia (CML) STI resistance

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

Patient history monitored with the CPA assay.
Example IV: Growing resistances in infectious diseases - Mycobacteria katG (Isoniazid)

- identification of multiresistant strains (MRS) in 2.5 hrs
- \( rpoB \) gen: 6 wt probes (compl. 81bp region)
  - 12 SNPs associated with RIF \( \text{res} \)
- \( katG \) gen: 315-Ser wt probe
  - 2 SNPs associated with INH \( \text{res} \)
- additional capture probes upon request (e.g. \( \text{inhA} \) gen etc.)

- 1 „duplex“ amplification
- 45 min protocol (excl. PCR)
- optional combination with quantitative Real-Time PCR
Example IV: Mycobacteria katG – Design
## Amplification – Melting Analysis wt/mt

<table>
<thead>
<tr>
<th></th>
<th>Sample Description</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative Control Tube [G]</td>
<td>26.89</td>
</tr>
<tr>
<td>2</td>
<td>S1142 (wt) without LNA</td>
<td>26.55</td>
</tr>
<tr>
<td>3</td>
<td>S1142 10 pmol LNA</td>
<td>31.23</td>
</tr>
<tr>
<td>4</td>
<td>S1143 (mt) without LNA</td>
<td>29.88</td>
</tr>
<tr>
<td>5</td>
<td>S1143 10 pmol LNA</td>
<td>35.77</td>
</tr>
<tr>
<td>6</td>
<td>S1142+1143 1:1</td>
<td>35.77</td>
</tr>
<tr>
<td>7</td>
<td>S1142+1143 1:10</td>
<td>38.73</td>
</tr>
<tr>
<td>8</td>
<td>S1142+1143 1:100</td>
<td>35.23</td>
</tr>
</tbody>
</table>

The diagram shows the melting analysis results for different samples, with the temperature (°C) at which melting occurs indicated for each condition.

*September 2005*
Amplification – Melting Analysis wt/mt

<table>
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<tr>
<th></th>
<th>Description</th>
<th>C</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NTC M. tub k.atG [50]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>S1142 (wt) ohne LNA</td>
<td>0.000E+00</td>
<td>26.03</td>
</tr>
<tr>
<td>3</td>
<td>S1142 10 pmol LNA</td>
<td>&lt;0.000E+00</td>
<td>&gt;41.00</td>
</tr>
<tr>
<td>4</td>
<td>S1143 (mt) ohne LNA</td>
<td>0.000E+00</td>
<td>26.55</td>
</tr>
<tr>
<td>5</td>
<td>S1143 10 pmol LNA</td>
<td>0.000E+00</td>
<td>31.29</td>
</tr>
<tr>
<td>6</td>
<td>S1142:1143 1:1</td>
<td>0.000E+00</td>
<td>31.23</td>
</tr>
<tr>
<td>7</td>
<td>S1142:1143 1:10</td>
<td>0.000E+00</td>
<td>35.77</td>
</tr>
<tr>
<td>8</td>
<td>S1142:1143 1:100</td>
<td>0.000E+00</td>
<td>38.73</td>
</tr>
<tr>
<td>9</td>
<td>S1142:1143 1:1000</td>
<td>0.000E+00</td>
<td>35.23</td>
</tr>
</tbody>
</table>

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Amplification – Melting Analysis wt/mt

1. NTC M. tub katG [58]
2. S1142 (wt) ohne LNA 0.000E+00 26.09
3. S1142 10 pmol LNA <0.000E+00 >41.00
4. S1143 (mt) ohne LNA 0.000E+00 26.55
5. S1143 10 pmol LNA 0.000E+00 31.29
6. S1142+1143 1:1 0.000E+00 31.23
7. S1142+1143 1:10 0.000E+00 35.77
8. S1142+1143 1:100 0.000E+00 38.73
9. S1142+1143 1:1000 0.000E+00 35.23

September 2005
Should all pregnant women be tested for their platelet PLA (Zw, HPA-1) phenotype? Br J Haematol. 1994 Jan;86(1):1-5.

About 97% of the population has the HPA-1a allele

Human platelet antigens (HPA) can be targets for antibody responses that cause life-threatening thrombocytopenia following platelet transfusions or pregnancy.

Search for fetal HPA-1b alleles in the maternal blood (HPA-1a background)
Locked Nucleic Acid opens new perspective