PCR development and troubleshooting

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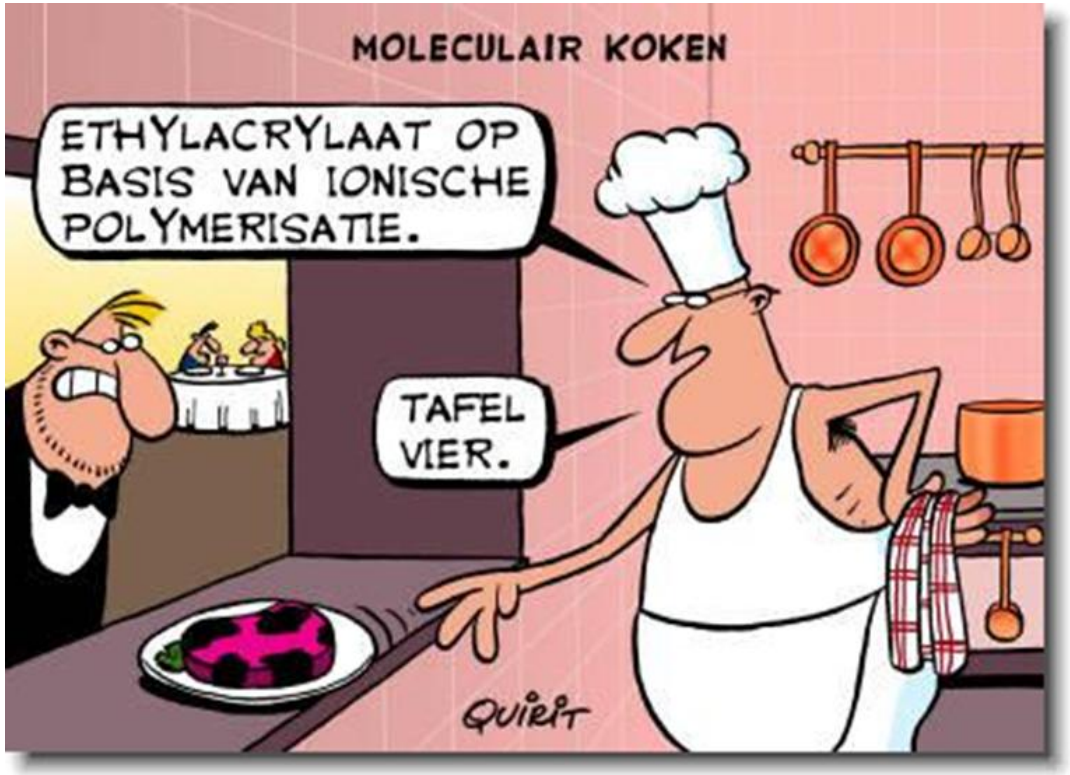
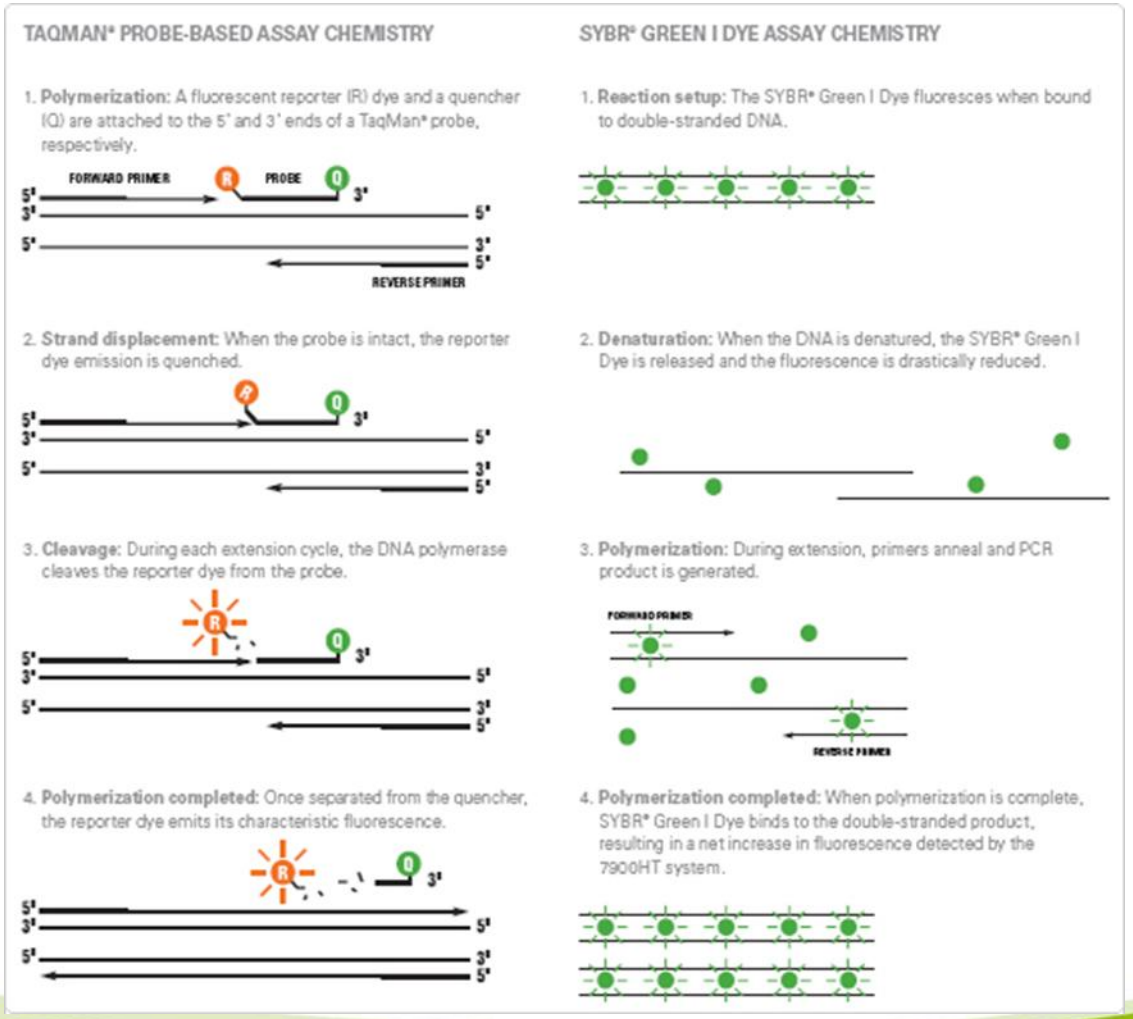
Science or handicraft?



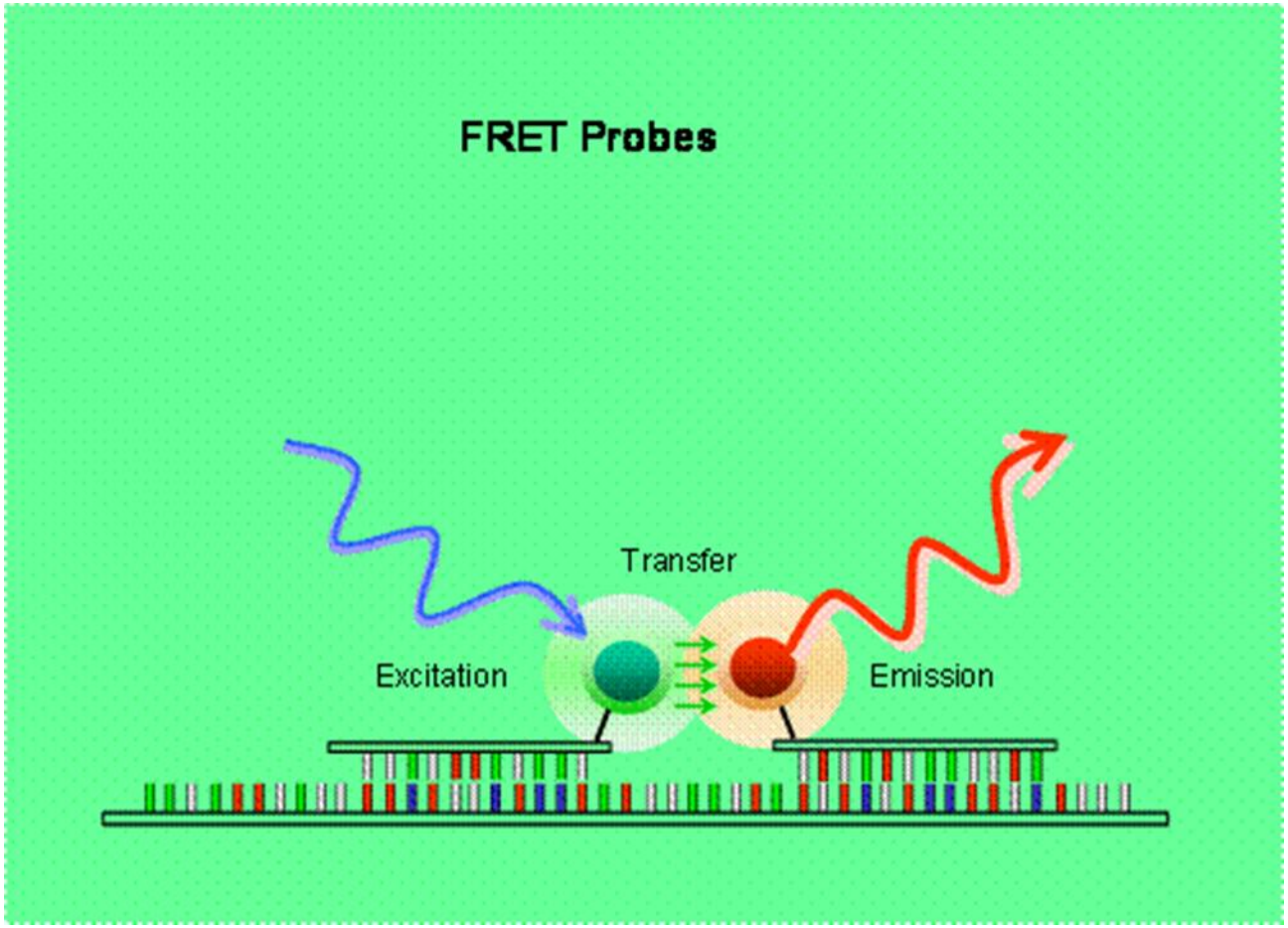
PCR development stages

- **Select PCR format and chemistry**
- **Select target**
- **Include/exclude regions**
- **Design primers and probe(s)**
- **Choose reporter and quencher**
- **Order oligo's**
- **Testing**

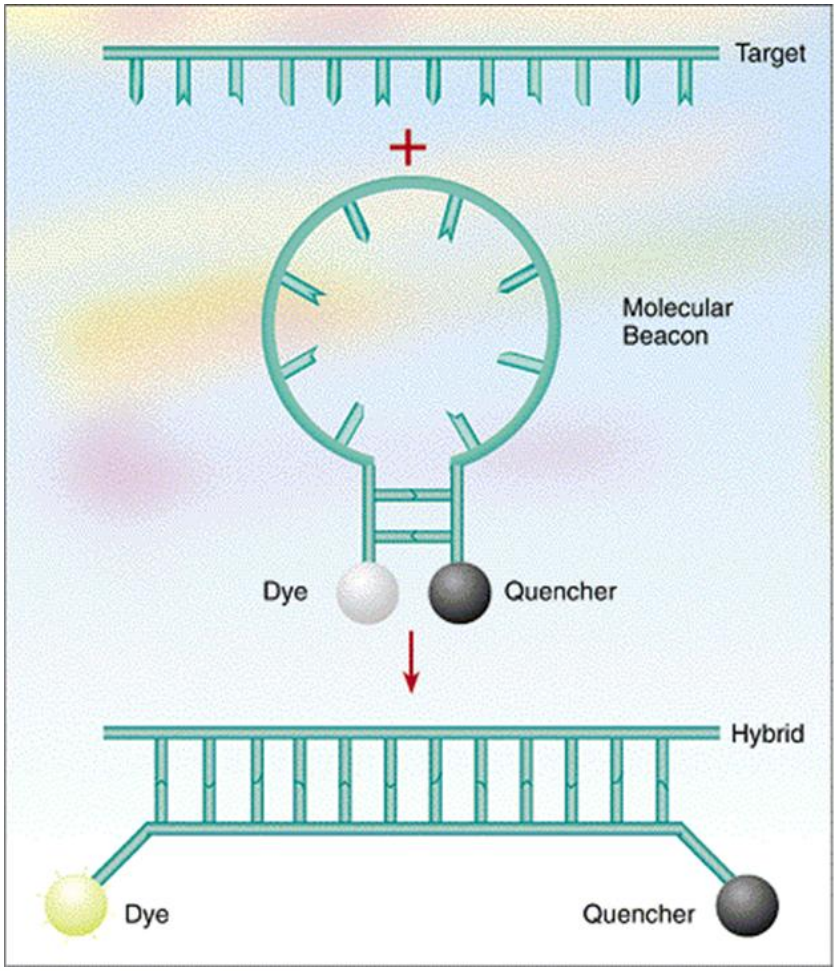
TaqMan and Sybr Green PCR



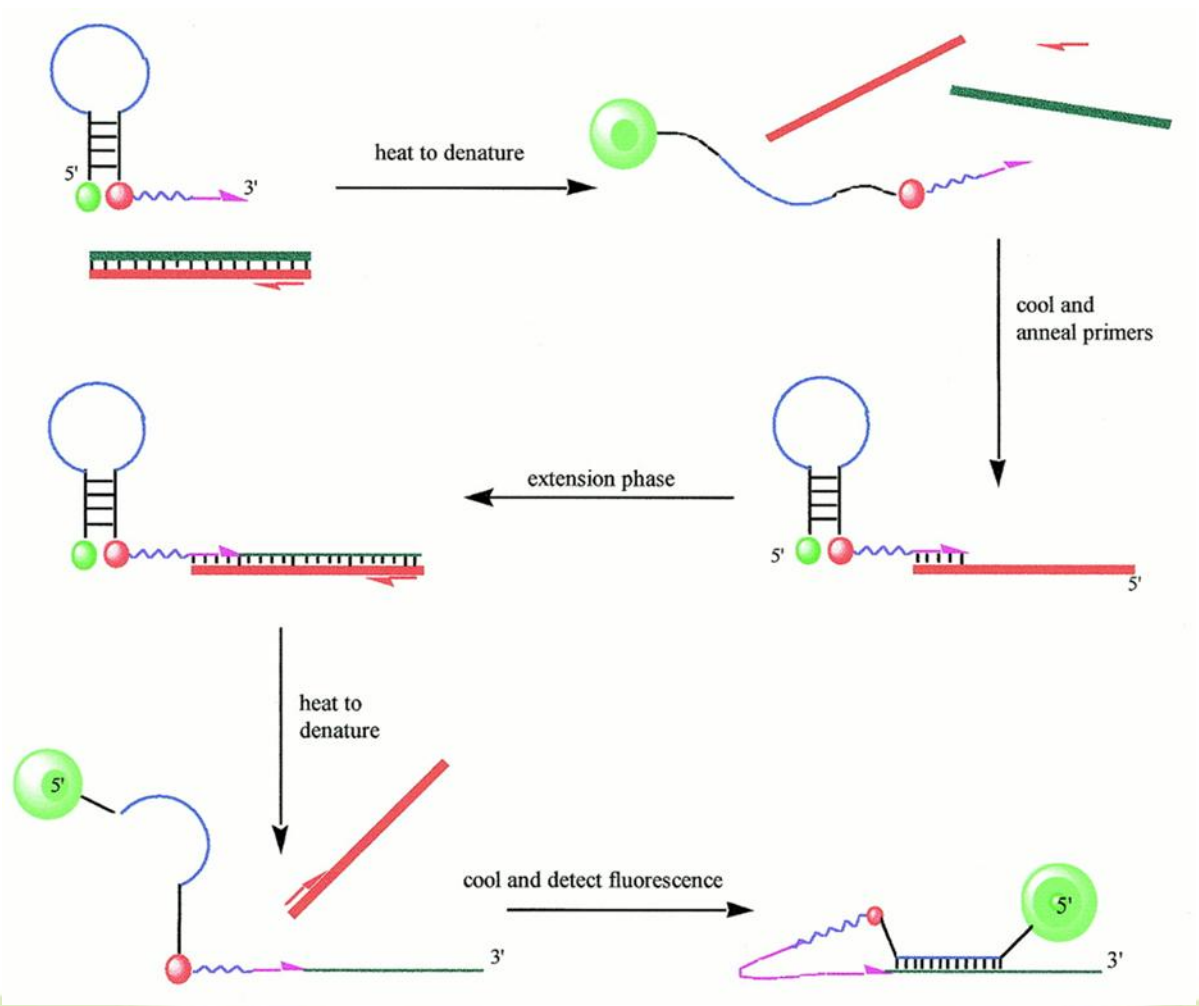
Hybridization probes



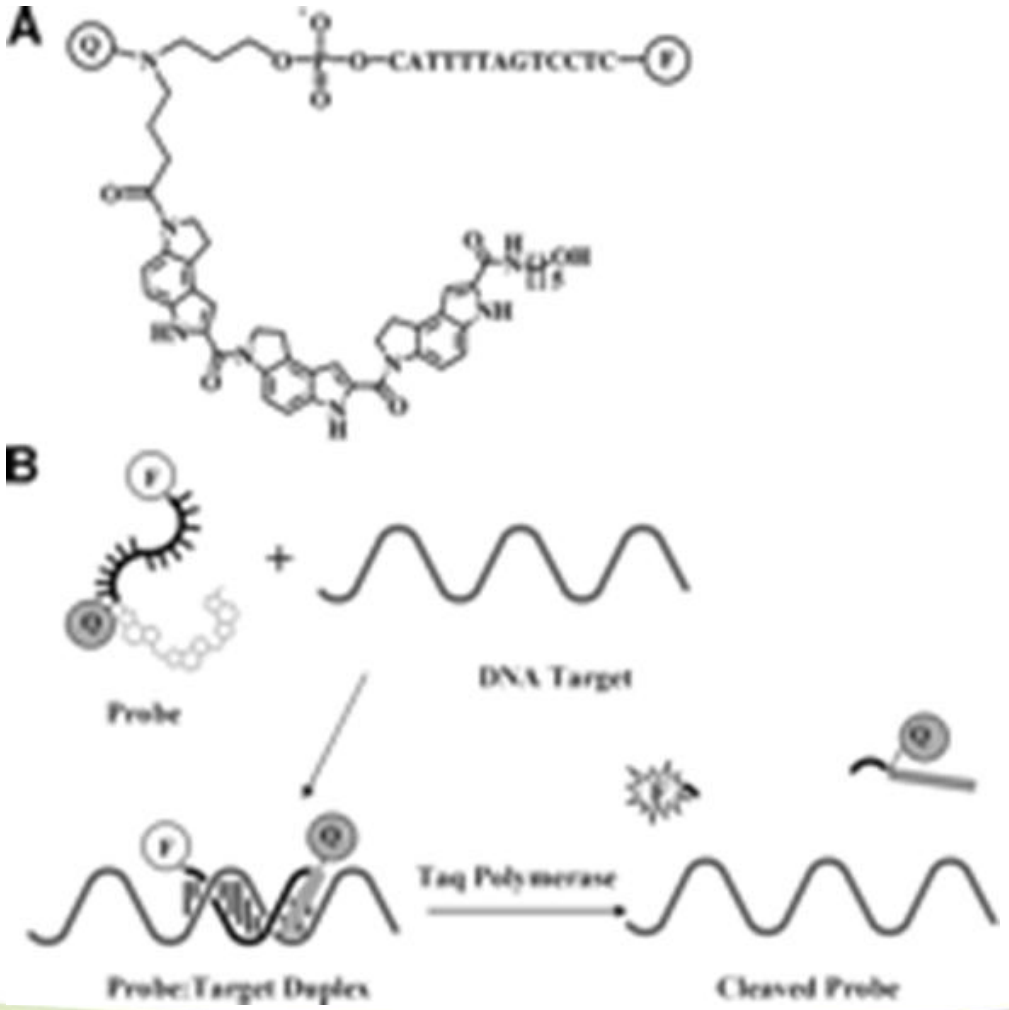
Molecular Beacons



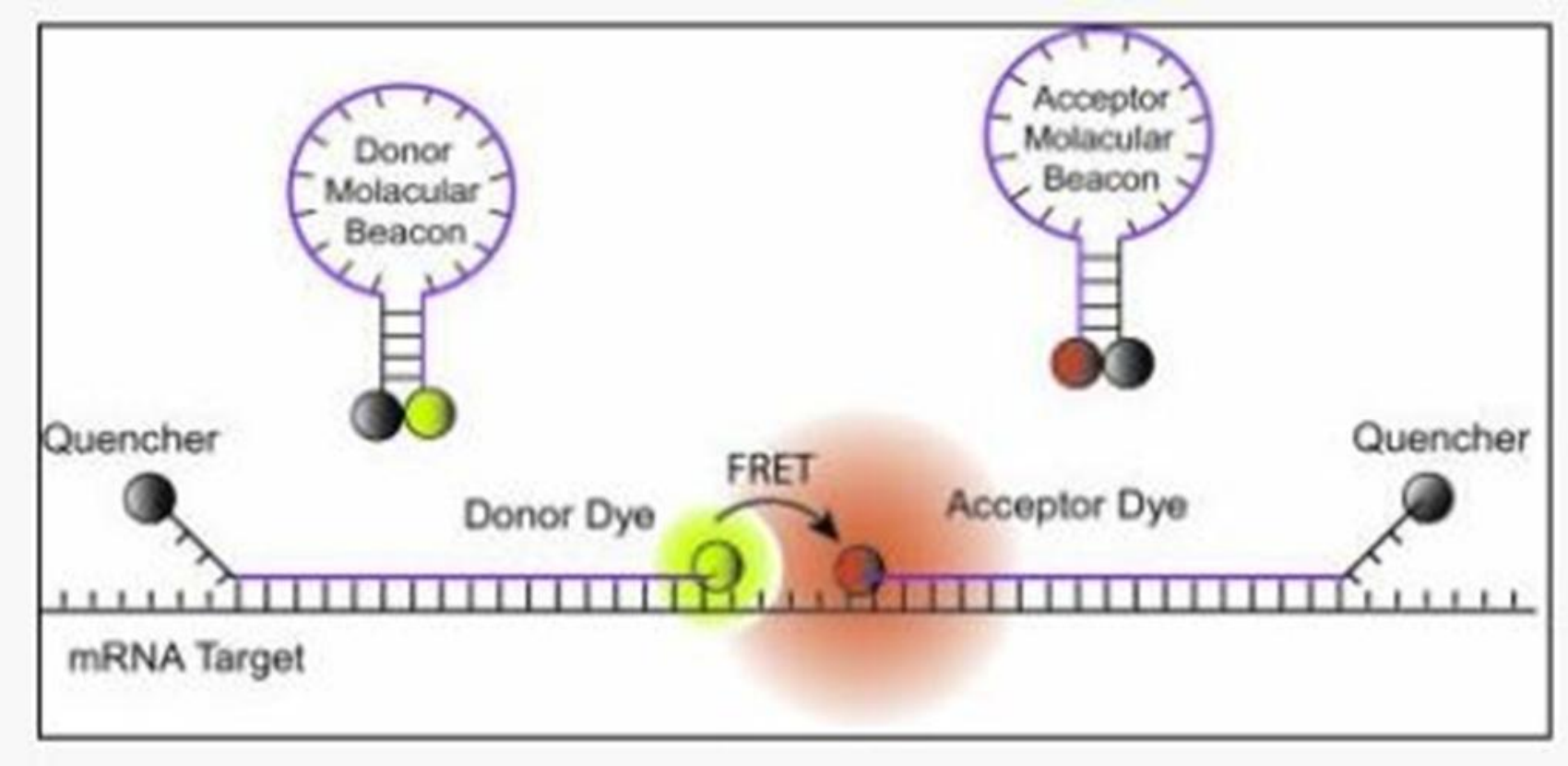
Scorpion primers



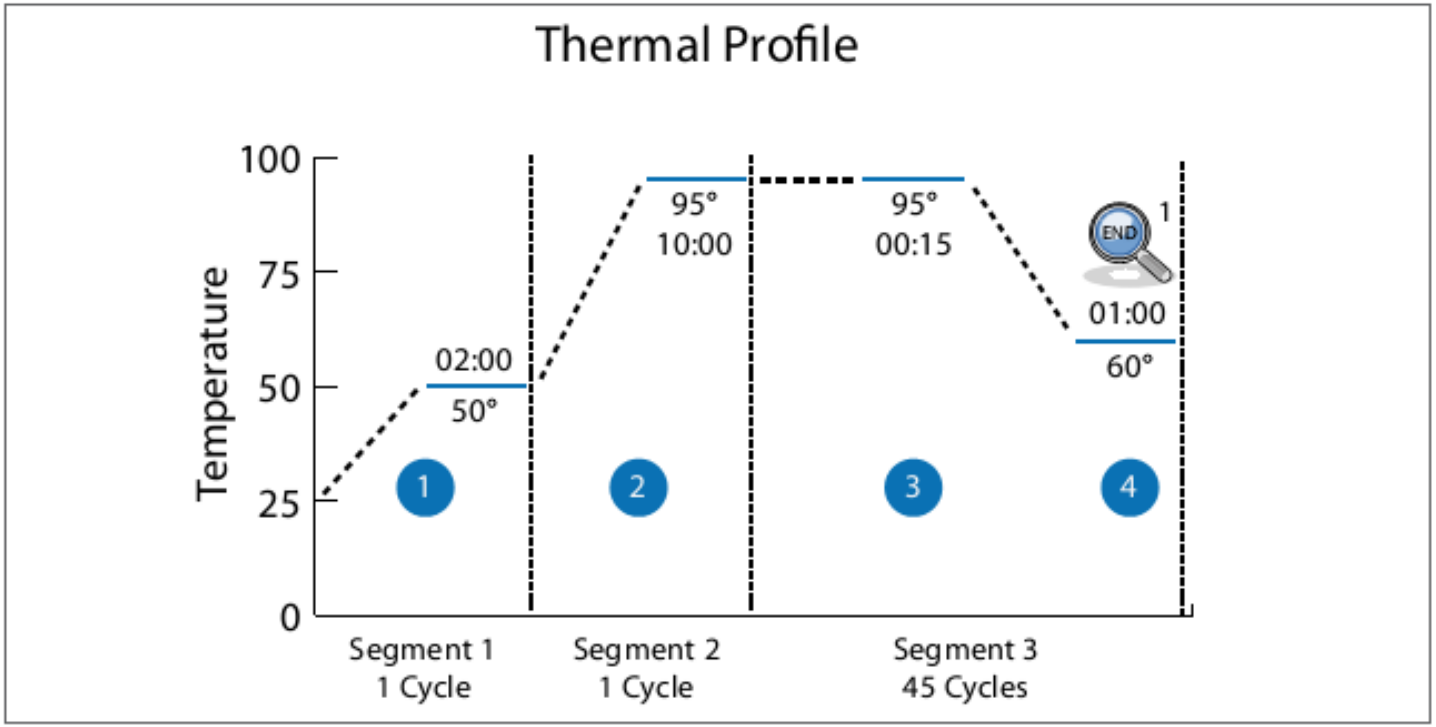
Minor Groove Binders



Combination of chemistries



Two step thermal profile



Target selection

- **micro-organisms**
- **gene expression profile**
- **translocations/fusion genes**
- **mutation/deletion/insertion**

Micro-organisms

- genus or species level
  - B. burgdorferi s.l.: different subspecies (genospecies)
  - enterovirus: different species and serotypes, ~rhinoviruses
- repeat sequences
  - T. gondii B1 repeat sequence (35 copies/genome) or AF146527 repeat sequence (250 copies/genome)
  - B. pertussis IS481 (100 copies/genome) but not 100% specific
- consensus or specific
  - length of consensus/conserved sequence
  - conserved sequence often rich in GC >> less efficient amplification
  - allow mismatches?

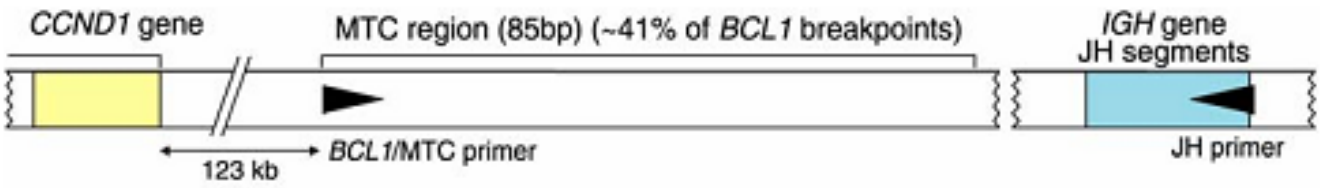
Gene expression profile

- specific for messenger RNA
  - intron spanning amplicon
  - primer directed to polyA
  - DNAse treatment
- presence of pseudogenes?
  - genomic BLAST alignment
- choice of housekeeping genes
  - stable expression level in assay conditions
  - increased number of HK genes increases confidence level

Vandesompele et al Genome Biology 2002
http://genomebiology.com/2002/3/7/research/0034

Translocations/fusion genes

size of breakpoint region



t(11;14) tube: 1 *BCL1* MTC primer + 1 JH primer

t(14;18) tube A: 2 *BCL2* MBR primers + 1 JH primer

t(14;18) tube B: 4 *BCL2* 3'MBR primers + 1 JH primer

t(14;18) tube C: 3 *BCL2* mcr primers + 1 JH primer

Mutation/deletion/insertion

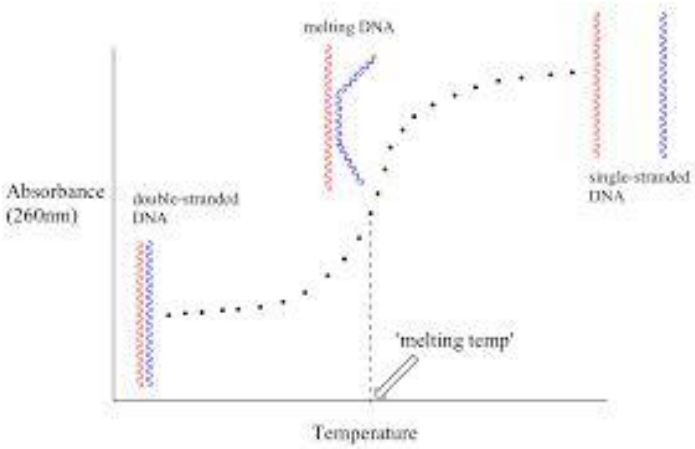
- hotspot or scattered
  - KRAS mutation hotspots in codons 12/13 (exon 2) and codon 61 (exon 3)
  - EGFR mutation scattered over exons 18 to 21
  - BRAF single mutation in codon 600
- DNA from FFPE tissue biopsies
  - fragmented and crosslinked
  - reduces the maximal amplicon size

Key design parameters

- **Length**
- **Tm**
- **%GC**
- **GC clamp / 3’ end stability**
- **Secondary structures** (also in template)
  - hairpin
  - self and cross dimers
- **Repeats and runs**

Tm estimation

- Tm= 4 x (# G + C) + 2 x (# A + T)
- Tm= 64.9 + 41 x (# G + C – 16.4)/length
- Tm= 81.5 + 16.6 x (log10[Na+] + [K+]) + 0.41 x (%GC) – 675/length
- Tm= {ΔH/ ΔS + R ln(C)} - 273.15
  - ΔH: change in enthalpy
  - ΔS: change in entropy corrected for salt conc.
  - R: universal gas constant
  - C: concentration of dsDNA/2



→ Use Tm calculator or primer design software

Primer design software

- **Primer Express**
- **Primer Premier**
- **Beacon Designer**
- **Oligo 7.0**
- **Primer 3.0 (or other freeware) >> Primer BLAST**
- **…**

On-line tools

NCBI: National Center for Biotechnology Information

- **GENBANK**: repository of nucleic acid and protein sequences
  http://www.ncbi.nlm.nih.gov/genbank/
- **BLAST**: Basic Local Alignment Search Tool
  http://blast.ncbi.nlm.nih.gov/
- **PRIMERBLAST**: test primers for specificity
  http://www.ncbi.nlm.nih.gov/tools/primer-blast/

University at Albany

- **UNAFOLD**: prediction of secondary structures (former mfold)
  http://mfold.rna.albany.edu/

T. gondii repetitive sequence

>gi|5916167|gb|AF146527.1|AF146527 Toxoplasma gondii repeat region
CTGCAGGGAGGAAGACGAAAGTTGTTTTTTTATTTTTTTTTCTTTTTGTTTTTCTGATTTTTGTTTTTTT
TGACTCGGGCCCAGCTGCGTCTGTCGGGATGAGACCGCGGAGCCGAAGTGCGTTTTCTTTTTTTGACTTT
TTTTTGTTTTTTCACAGGCAAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAAGGGGACTA
CAGACGCGATGCCGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGAAGGCGAGGGTGA
GGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGAGAGGGAGAAGATGTTTCCGGCTTGGCTGC
TTTTCCTGGAGGGTGGAAAAAGAGACACCGGAATGCGATCCAGACGAGACGACGCTTTCCTCGTGGTGAT
GGCGGAGAGAATTGAAGAGTGGAGAAGAGGGCGAGGGAGACAGAGTCGGAGGCTTGGACGAAGGGAGGAG
GAGGGGTAGGAGAGGAATCCAGATGCACTGTGTCTGCAG

EXERCISE

- look for sequence in Genbank
- align with similar sequences using BLAST
- evaluate which regions to include or exclude

Probe design guidelines

- G-C content between 20 and 80%
- Avoid runs of an identical nucleotide. Runs of four or more Gs should be avoided.
- No G on the 5´end
- More C than G
- Melting temperature (Tm) should be 68 to 70°C when using Primer Express software.

EXERCISE

- select an interesting probe binding region
- design a probe compliant with design guidelines
- check probe specificity using BLAST

Primer design guidelines

- Choose the primers after the probe
- Put the primers as close as possible to the probe without
- overlapping
- G-C content between 20 and 80%
- Avoid runs of an identical nucleotide. Runs of four or more Gs should be avoided
- Tm of each primer should be 58 to 60°C.
- The five nucleotides at the 3´end of each primer should have no more than two G and/or C bases.

EXERCISE

- select an interesting primer binding region
- design forward and reverse primer compliant with design guidelines
- check primer specificity using primer BLAST

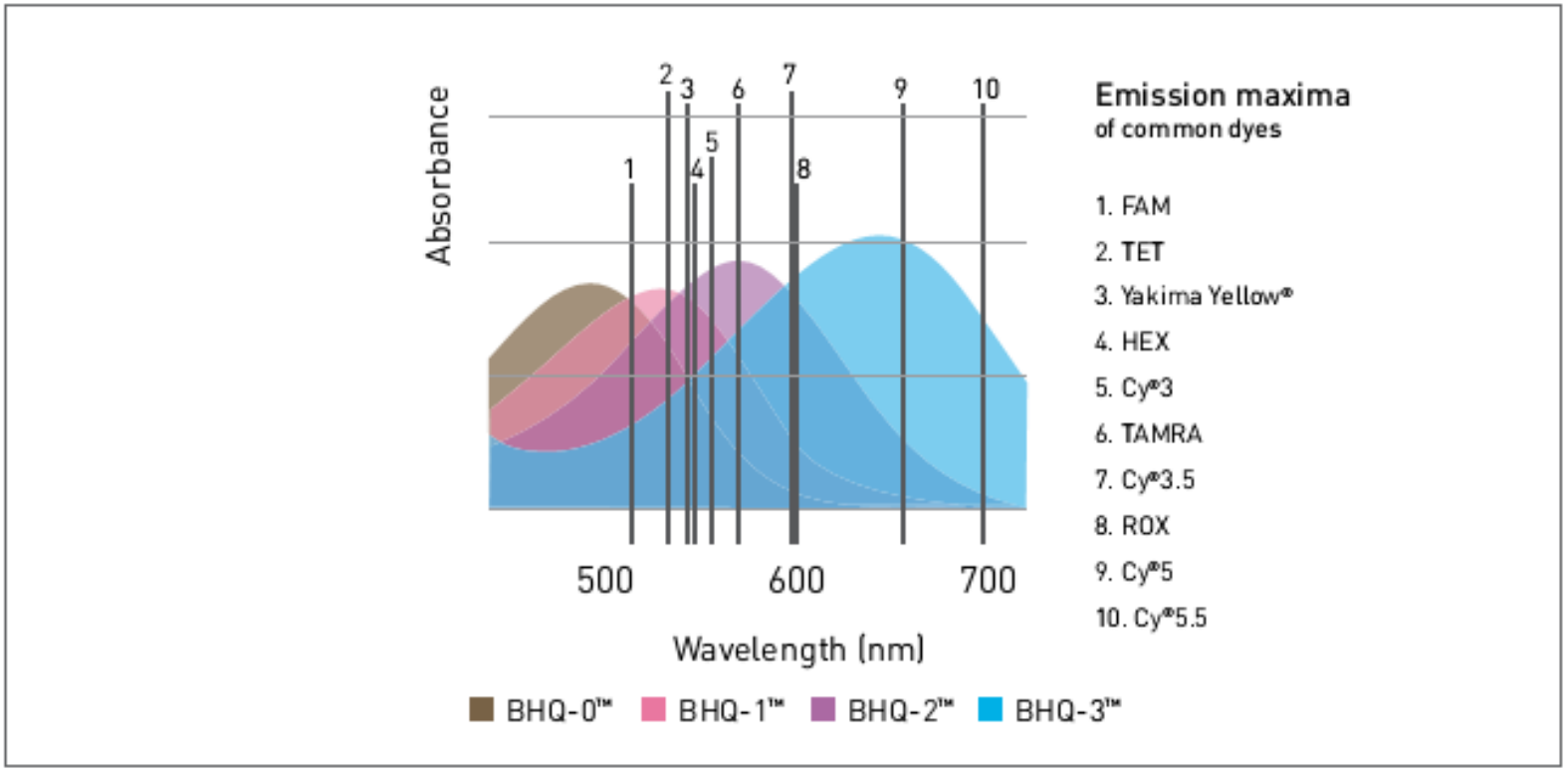
Reporters and quenchers

- **REPORTER**
  - compatible with the thermocyclers optics
  - high fluorescence
  - narrow spectrum and well separated
- **QUENCHER**
  - quencher compatible with fluorochrome
  - high signal/noise ratio
  - singleplex FAM TAMRA (economical)
  - multiplex dark quenchers

Thermocycler compatibility

Thermocycler	Channel 1	Channel 2	Channel 3	Channel 4	Channel 5	Channel 6	Channel 7
GeneAmp® SDS 5700	FAM						
ABI Prism® SDS 7000	FAM	YY/VIC/JOE	DFO/NED	ROX	–		
ABI Prism® SDS 7700	FAM	YY/VIC/TET/JOE	DFO/NED	ROX	–		
ABI Prism® SDS 7900 HT	FAM	YY/VIC/TET/JOE	DFO/NED	ROX	–		
ABI Prism® SDS 7300	FAM	YY/VIC/JOE	DFO/NED	ROX	–		
ABI Prism® SDS 7500	FAM	YY/VIC/JOE	DFO/NED/Cy®3	ROX/TR	Cy®5	–	
StepOne®	FAM	YY/VIC/JOE	ROX	–	–		
StepOnePlus®	FAM	YY/VIC/JOE	DFO/NED	ROX	–		
Cycler iQ®	FAM	YY/VIC/HEX/TET/ Cy®3	DFO/NED	ROX	Cy®5		
My iQ®	FAM	–					
iQ5	FAM	YY/VIC/HEX/TET	DFO/NED	ROX/TR	Cy®5	–	
CFX 96®	FAM	YY/VIC/HEX	ROX / TR	Cy®5	Cy®5.5 / Quasar 705		
MiniOpticon®	FAM	YY/VIC/HEX/TET	–	–	–		
DNA Engine Opticon® 1	FAM	–		–	–		
DNA Engine Opticon® 2	FAM	YY/VIC/HEX/TET	–	–	–		
Chromo 4®	FAM	YY/VIC/JOE/TET	DFO/NED/ROX/TR	Cy®5	–		
Mx3000P® (choice of 4 filtres)	FAM	TET	YY/VIC/JOE/HEX	Cy®3	DFO/NED	TR/ROX	Cy®5
Mx3005P® (choice of 5 filtres)	FAM	TET	YY/VIC/JOE/HEX	Cy®3	DFO/NED	TR/ROX	Cy®5
Mx4000® (choice of 4 filtres)	FAM	TET	YY/VIC/JOE/HEX	Cy®3	DFO/NED	TR/ROX	Cy®5
Mastercycler®ep realplex2	FAM	YY/VIC/JOE/HEX/TET	–	–	–		
Mastercycler®ep realplex4	FAM	YY/VIC/JOE/HEX/TET	DFO/TAMRA	ROX	–		
LightCycler® 1.5	FAM	YY/VIC/JOE/HEX/TET	LC Red 705/ Cy®5	–			
LightCycler® 2	FAM	LC Red 640/ROX	LC Red 610	LC Red 640	LC Red 670	LC Red 705	
LightCycler 480®	FAM	YY/VIC/HEX	LC Red 610	LC Red 640/ROX	Cy®5	LC Cyan 500	
Smartcycler®1	FAM	YY/VIC/TET/JOE/ Cy®3	TR	Cy®5			
Smartcycler®2	FAM	Cy®3/ TET	TR/ROX	Cy®5			
Rotor-Gene™ 2000 / 3000	FAM	YY/VIC/JOE/TET	ROX/Cy®3.5	Cy®5			
Rotor-Gene™ 6000	FAM	YY/VIC/JOE/TET/HEX	ROX/TR/Cy®3.5	Cy®5	Alexa®680/Atto680	Alexa® Fluor-350	

Quenchers fits reporter



Probe synthesis

- **From 3’ to 5’ end**
- **Fluorochrome at 5’ end or internal**
- **automated coupling**
  - phosphoramidite
  - only at 5’end
- **manual coupling**
  - some fluorochromes
  - also internal labelling possible
  - low yield
  - more expensive

Ordering oligo's

- **Eurogentec**

  http://www.eurogentec.com/eu-home.html
- **Tibmolbiol**

  http://www.tib-molbiol.com/
- **Biolegio**

  http://www.biolegio.com/
- **Eurofin (Operon)**

  http://www.eurofinsgenomics.eu/
- **Life Technologies (Invitrogen)**

  http://www.invitrogen.com/
- **…**

The proof of the pudding is in the eating