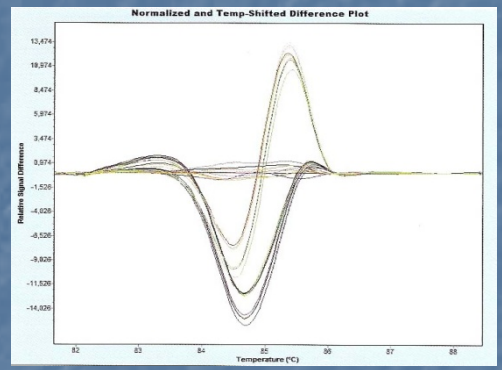
BRCA screening by HRM on the LightCycler® 480



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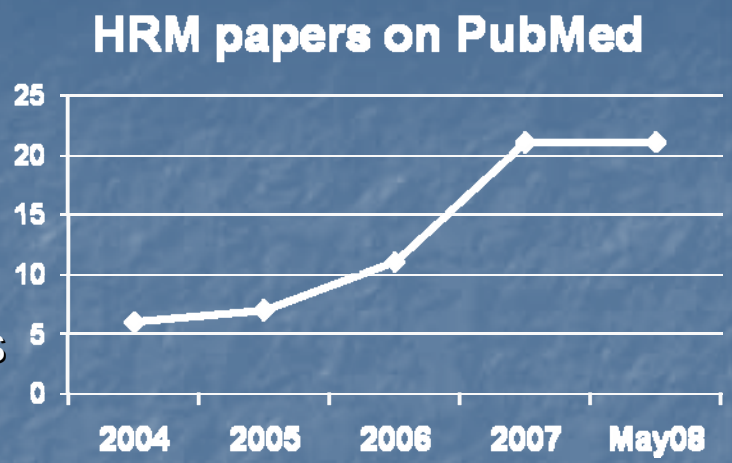
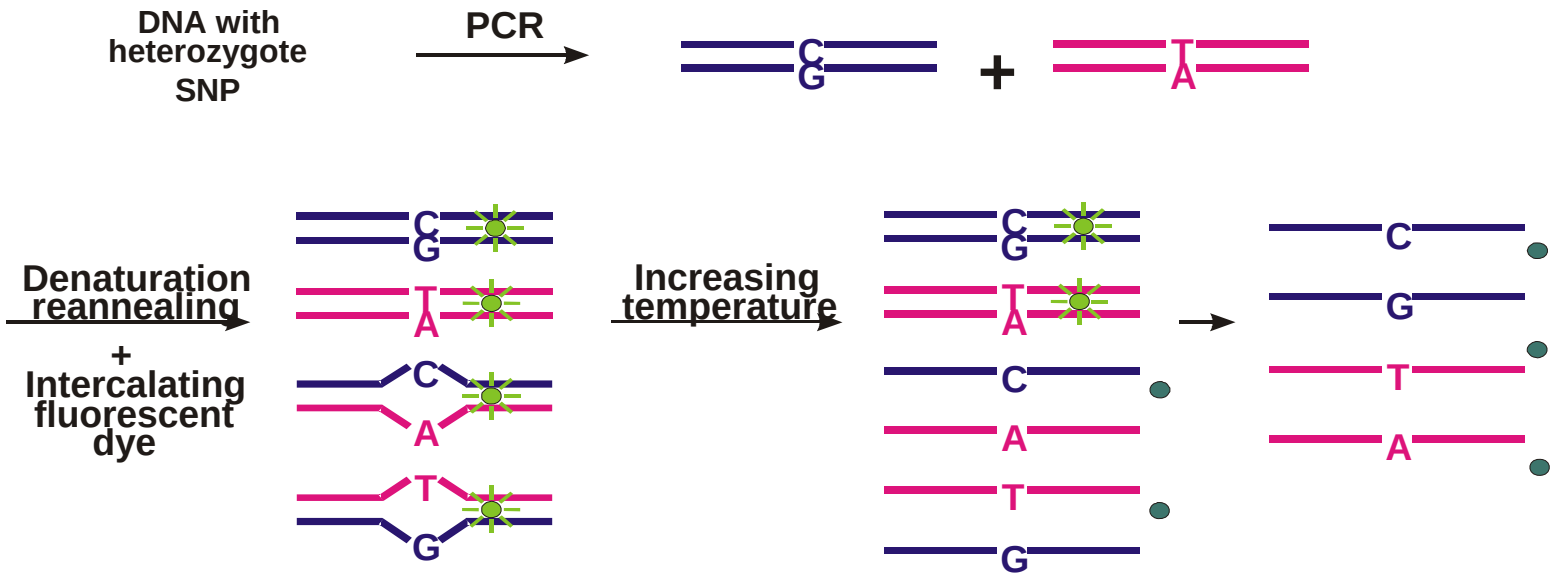
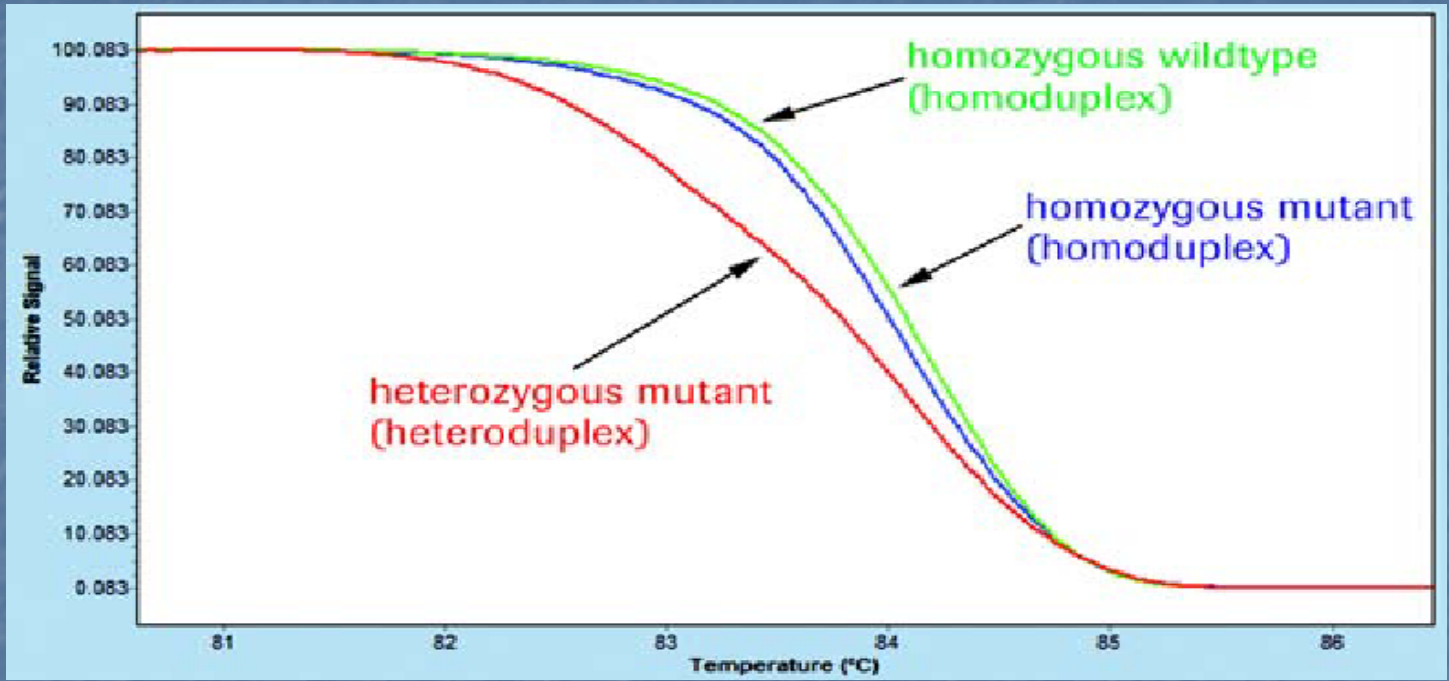
HRM

- What is HRM?
  - High resolution melting
  - Mutation scanning
  - Based on difference in melting patterns between homo- and heteroduplexes





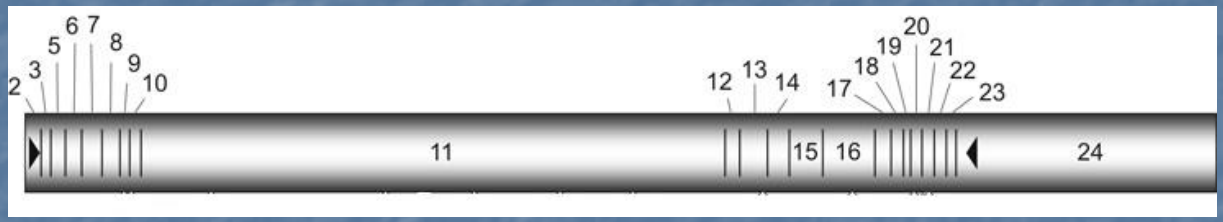
HRM



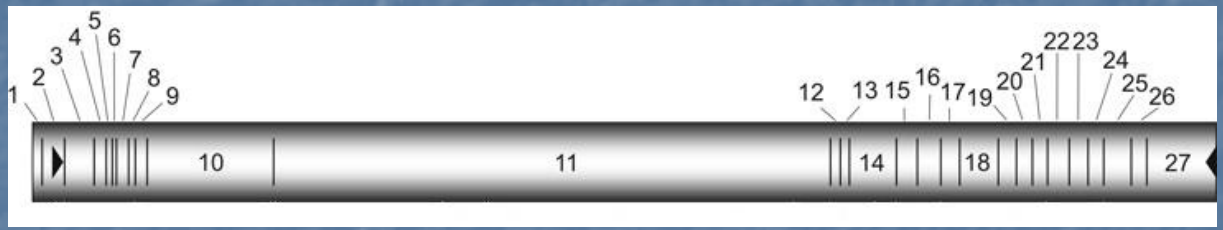
- Applications
  - Mutation scanning (screening for unknown sequence variations)
  - Genotyping (detection of known SNPs using unlabeled probes)
  - Mutation scanning + genotyping (combination)
  - Methylation analysis

BRCA1 and BRCA2 screening

- Very challenging genes
  - BRCA1: 22 coding exons



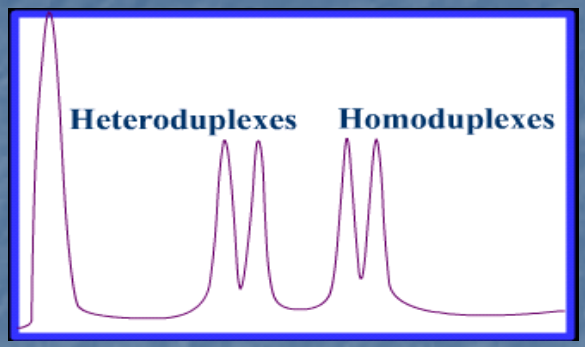
  - BRCA2: 26 coding exons

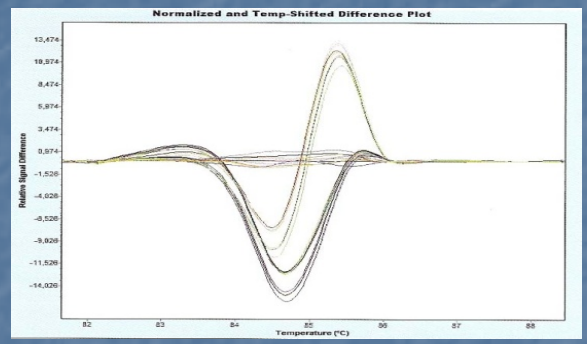


- Lower than average GC content
- No mutation hot spots (insertions, deletions, duplications)
- Increasing number of clinical samples

➡ 2 strategies: **direct sequencing** or **2-tier strategy**

DHPLC vs HRM





- **Good performance …**
  - high sensitivity (>95%)
  - high specificity (>98%)

- **… but some limitations**

Throughput
- Serial system
- Separation of duplexes
- multiple Tm

Maintenance
- Daily
- Weekly
- Monthly

- Cheaper system
- No expensive consumables
- “maintenance-free”
- Closed-tube system

- No separation of duplexes
- 10-15min melt

Much faster !!!

LC480 validation study: Setup

- **Extensive EuroGentest validation**
  - Collaboration (Leuven, Leiden, Geneva)
  - Many samples – different types of sequence variations
  - Inter-laboratory parameters

- **3 phases**
  - PCR optimization
  - Mutation detection optimization
  - Blind study

- **Aim**
  - A complete primer set for BRCA1 and BRCA2 screening
  - Power to determine a sensitivity of at least 99%
  - Easy implementation in a diagnostic setting

PCR optimization

- Primers:
  - Based on existing primer sets for dHPLC or sequencing
  - SNPcheck (NGRL Manchester)
  - Fragment length (~ 200 – 400bp)
  - M13 tags → universal primers → streamlined sequencing

BRCA1: 42 amplicons
BRCA2: 61 amplicons

- PCR

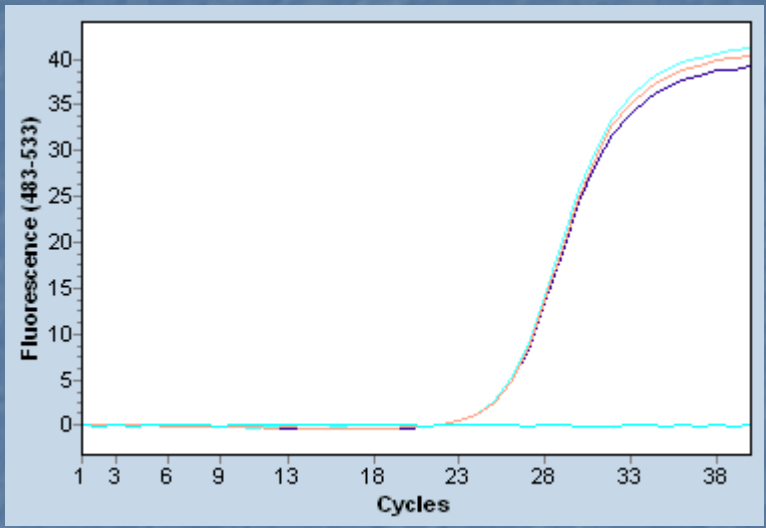
Criteria

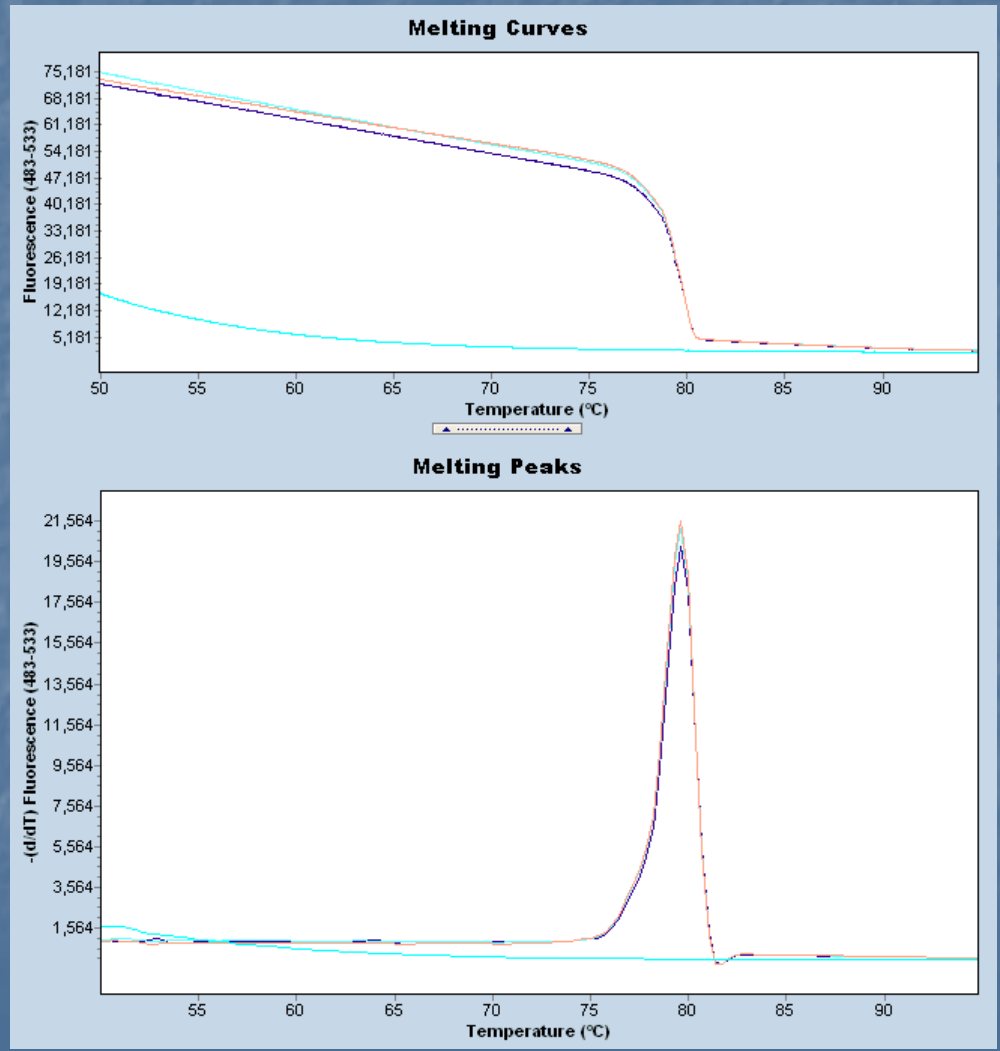
- sigmoid shape of real-time curve
- Cp < 28
- specific bands on 2% agarose
- < 3 melting domains

Starting conditions

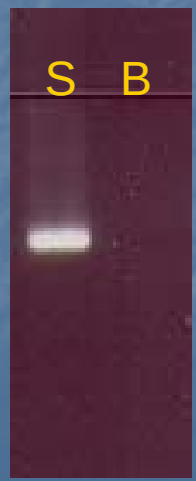
- 10 µl reactions
- 1x HRM master (Roche Applied Science)
- 2 or 3 mM magnesium
- 250 nM primers
- 20 ng template DNA
- same annealing temperature if possible
- 40 cycles

PCR optimization: starting conditions

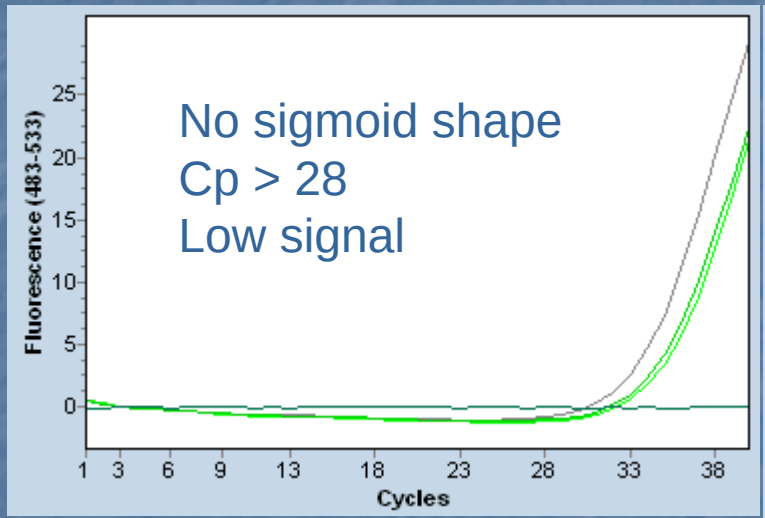




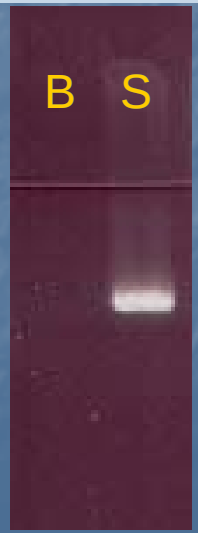
BRCA2 exon 2
Ta: 60°C
2mM Mg

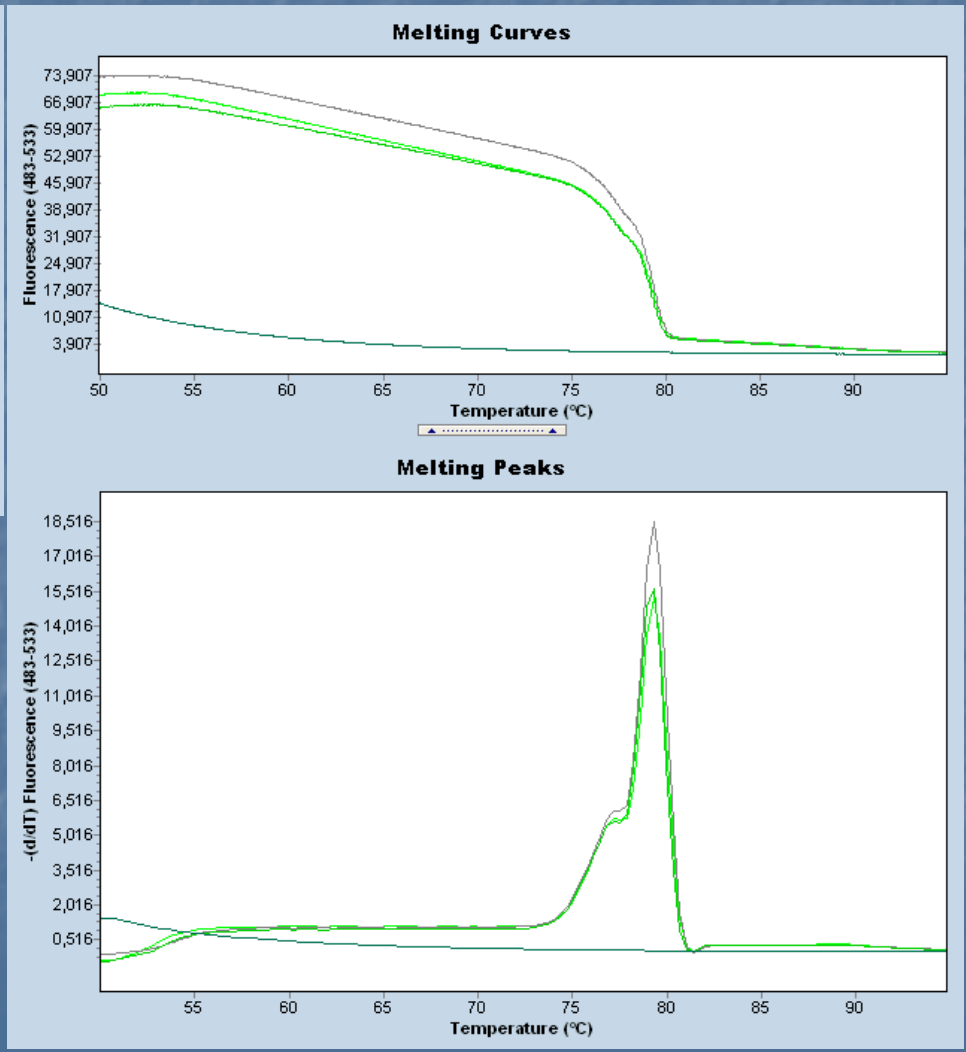


PCR optimization: starting conditions

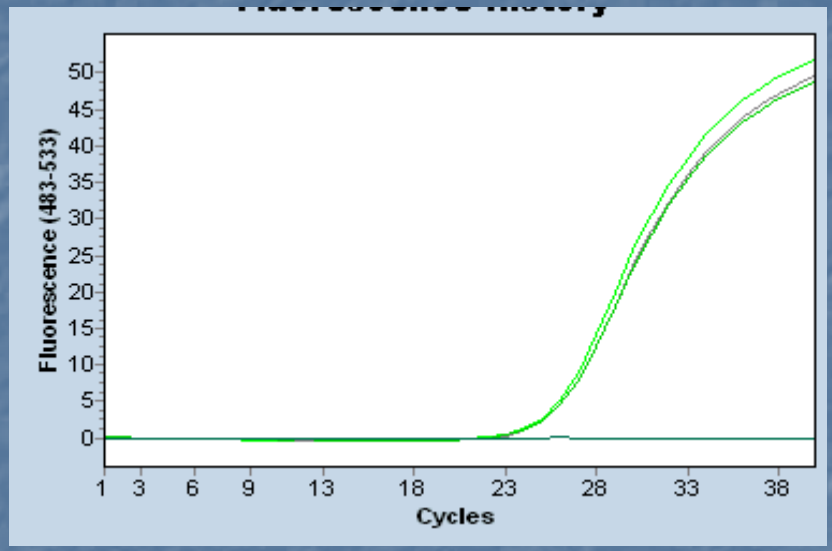


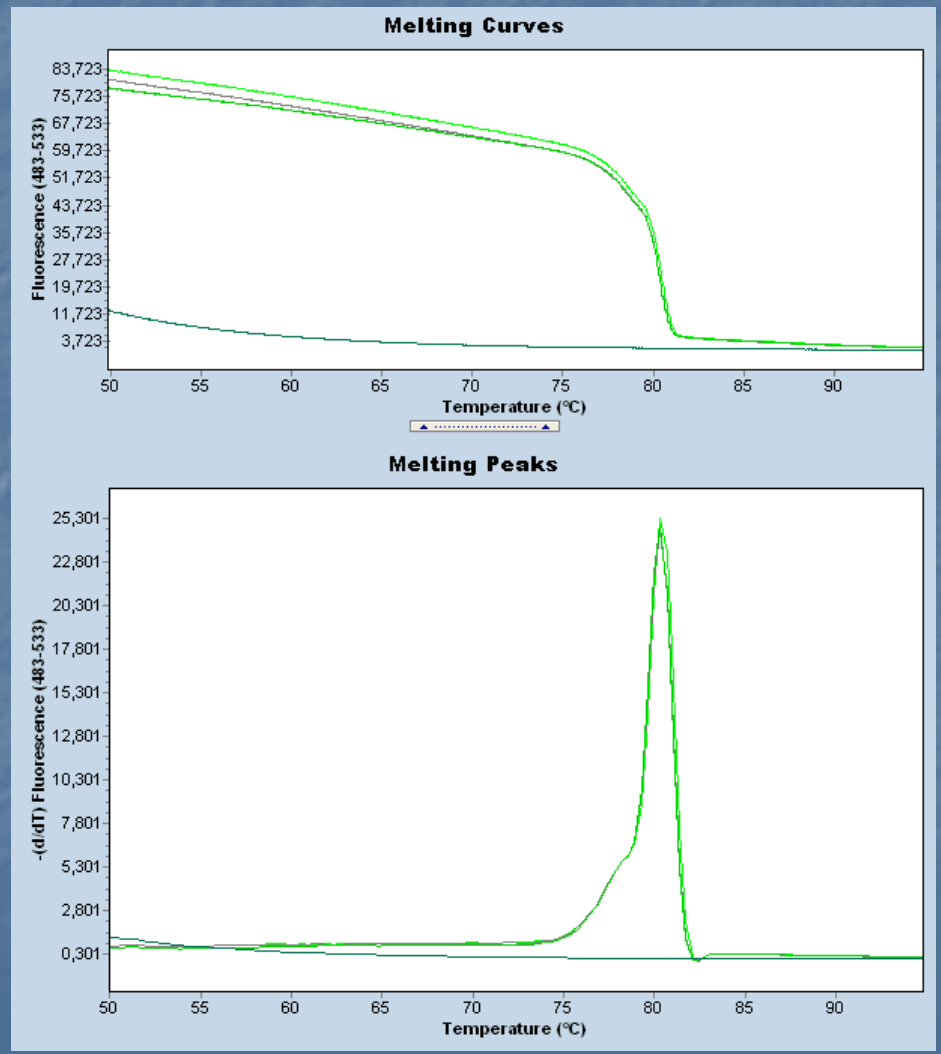
BRCA2 exon 3
Ta: 60°C
2mM Mg





PCR optimization: fine-tuning





BRCA2 exon 3
Ta: 57°C
3mM Mg

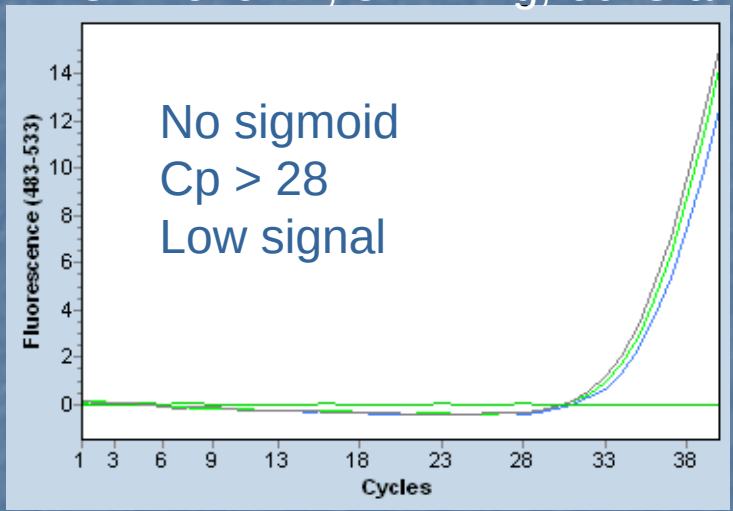
Critical parameters

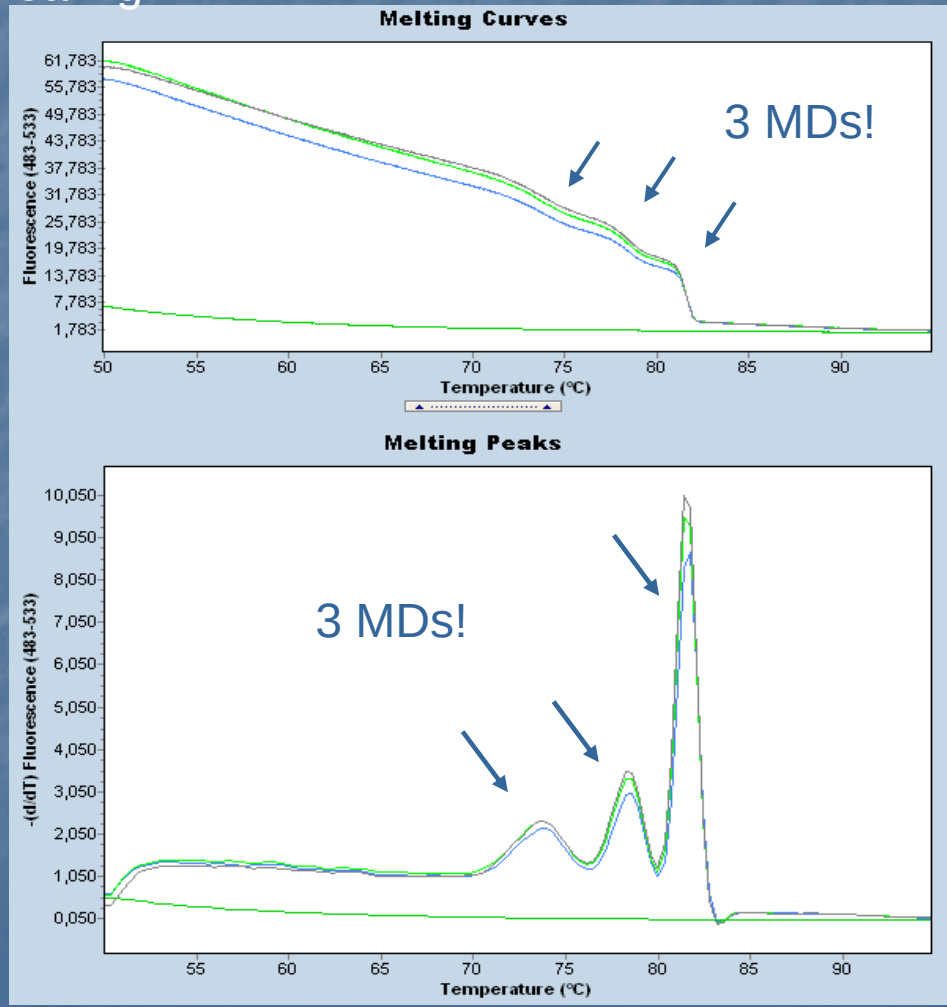
Annealing temperature

Magnesium concentration

PCR optimization: redesign amplicon

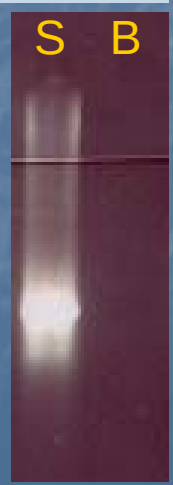
BRCA2 exon 7, 3mM Mg, 60°C annealing





BRCA2 exon 7
Ta: 60°C
3mM Mg

Smeared sample



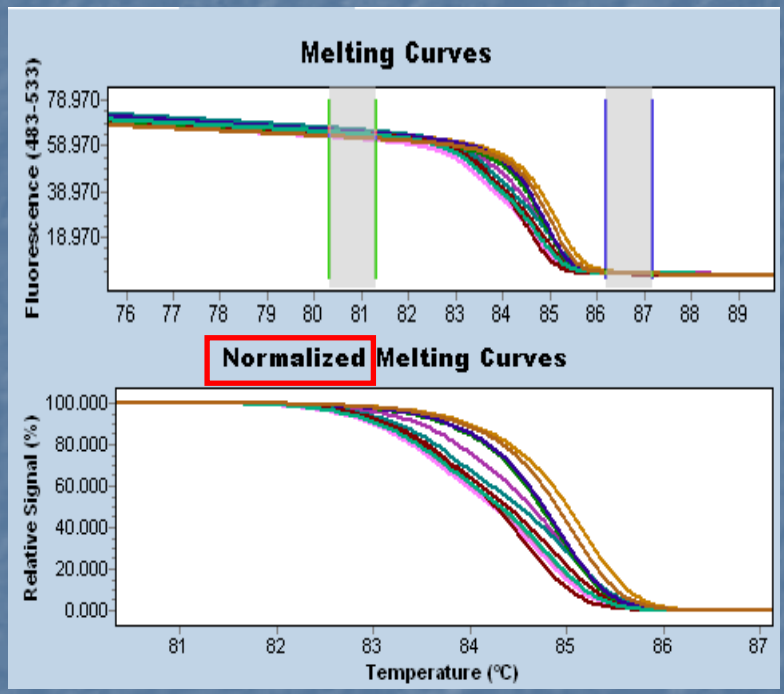
Redesign primer!

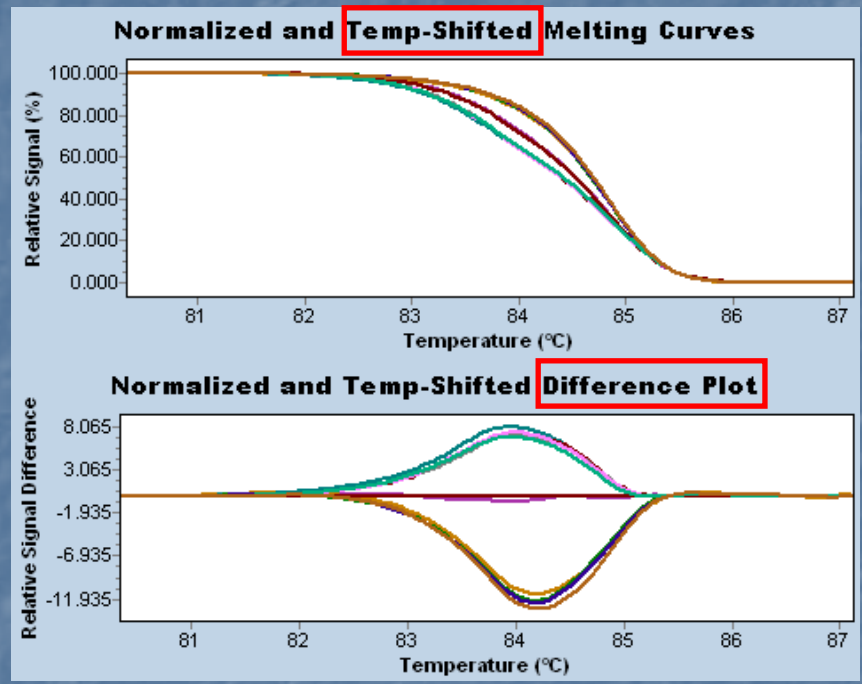
PCR optimization: Summary

- 103 amplicons
  - BRCA1: 42
  - BRCA2: 61
- fixed annealing temperatures
  - BRCA1: 4 blocks of at least 6 amplicons (59°C, 62°C, 63°C, 65°C)
  - BRCA2: all at 57°C
- all amplicons met required criteria

Mutation detection optimization

- Gene scanning software (v1.3 → v1.5)

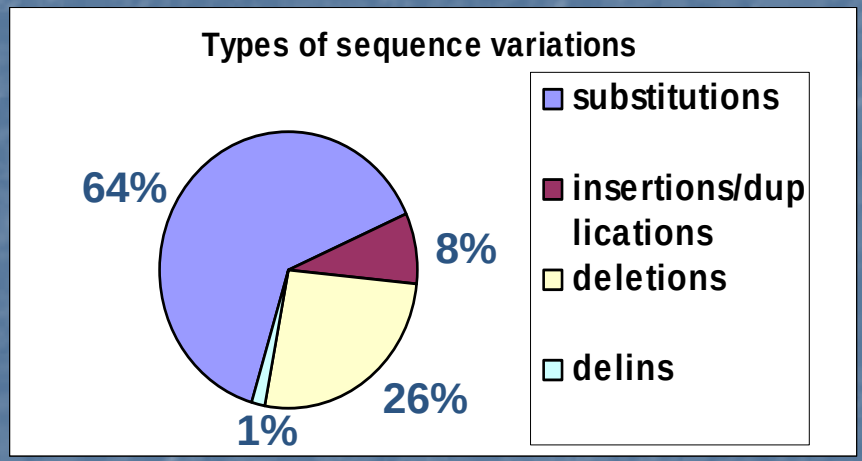




- Critical parameters for mutation detection
  - Pre- and post normalization interval settings
  - Sensitivity setting

Mutation detection optimization

- Setup
  - Set of ~ 300 DNA samples with known sequence variations



  - Determine optimal settings for each amplicon
    - Normalization strategy: 1°C intervals right before and after melt region
    - Sensitivity: range without false negatives (and false positives)

Mutation detection optimization: Example

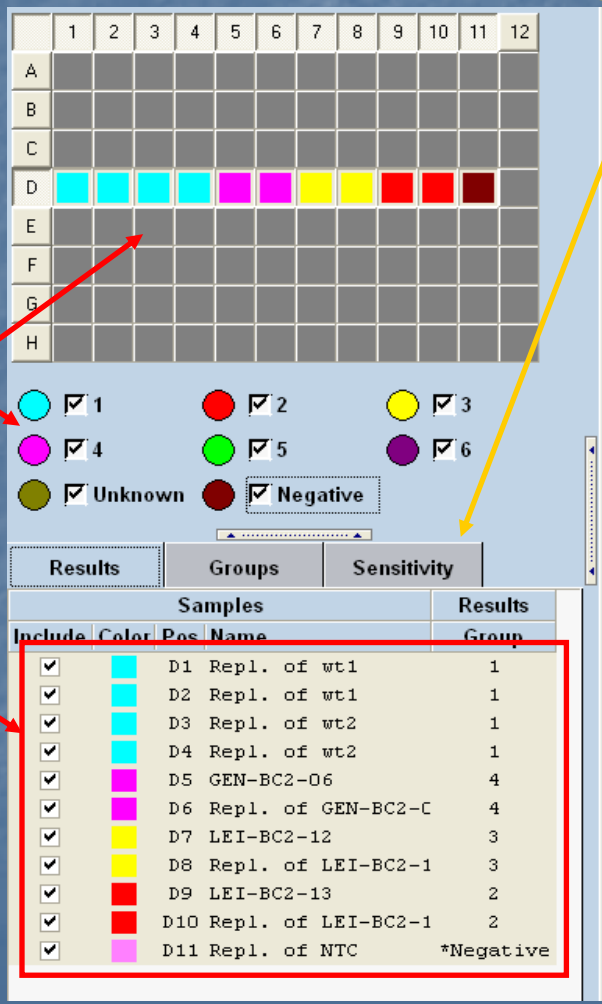
BRCA2 exon 9

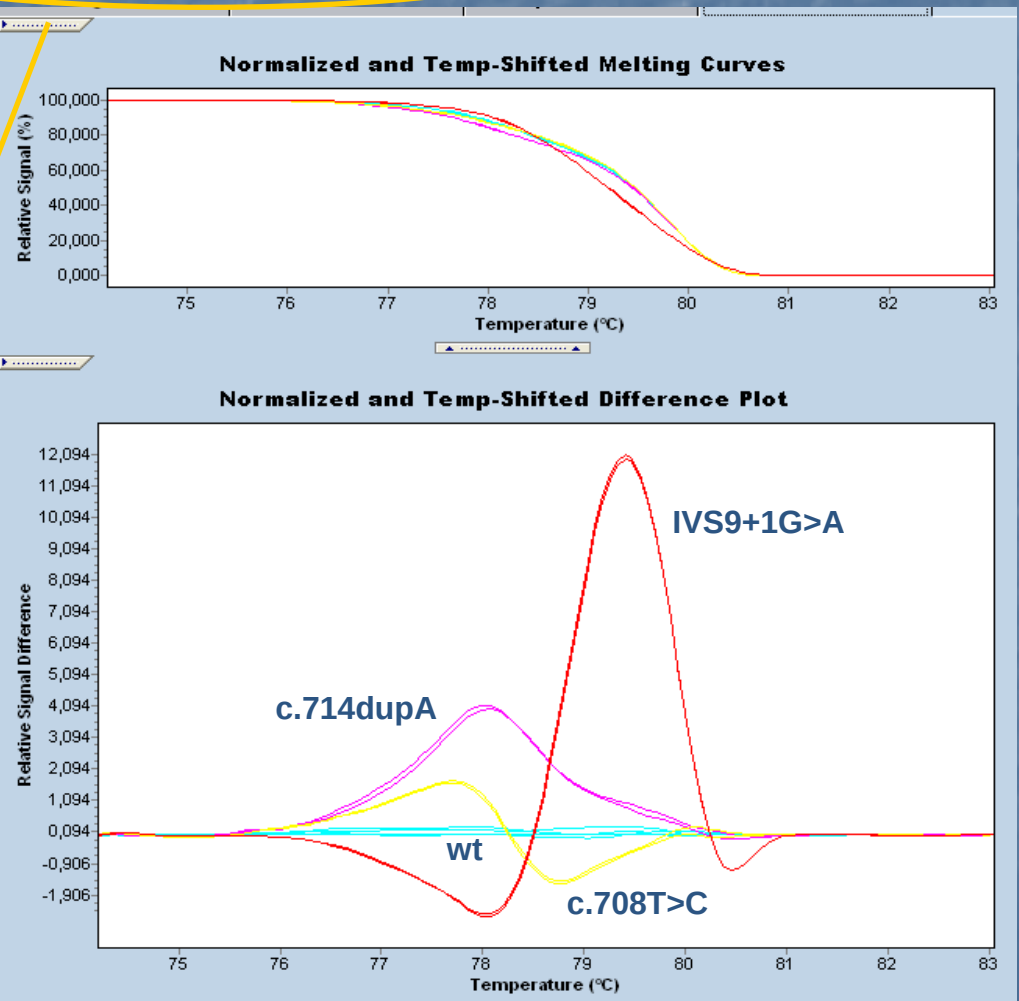
Sensitivity range = 0.63 – 1.00

OK

Automatic grouping function

Sample ID





Mutation detection optimization: Summary

- All analyses performed on software v1.3
- Detection rates:
  - BRCA1: 94%
  - BRCA2: 97%
- Fixed normalization intervals
- Variable sensitivity ranges

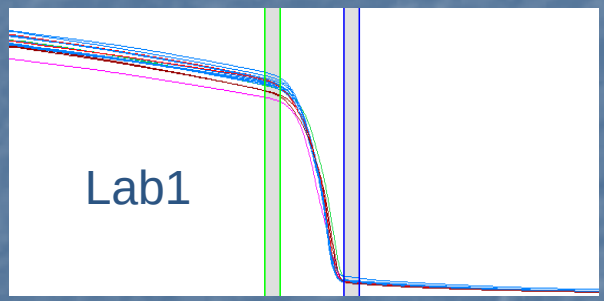
Blind study

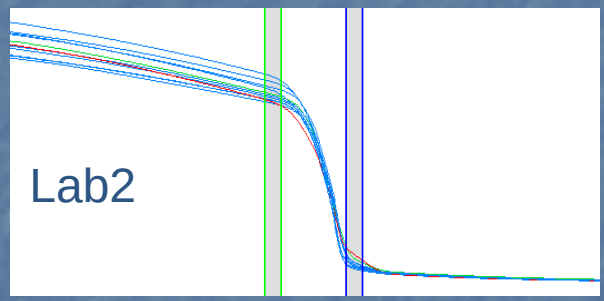
- Aim
  - determine sensitivity and specificity of HRM
  - ~ 300 positive control samples (~ 150 per gene)
  - Power to reach a sensitivity of at least 99% (CI: 95%)
  - At least as many wild type samples to have equal power for specificity

- Setup
  - Random distribution of samples (11 per amplicon + 1 ntc)
  - Random distribution of amplicons over 3 labs → inter-lab variability
  - Blind and automatic analysis
  - Pre-defined and optimized normalization and sensitivity settings
  - Upgrade from Software 1.3 to 1.5

Blind study: Results

- Normalization settings
  - Poor reproducibility between laboratories
  - Shift in normalization intervals





- Sensitivity settings
  - Fixed at 0.60 for most amplicons with software v1.5
  - some amplicons higher sensitivity setting

- Preliminary sensitivity
  - BRCA1: > 97% (96% with v1.3)
  - BRCA2: > 99% (96% with v1.3)
- Preliminary specificity
  - BRCA1: > 92% (88% with v1.3)
  - BRCA2: > 77% (82% with v1.3)

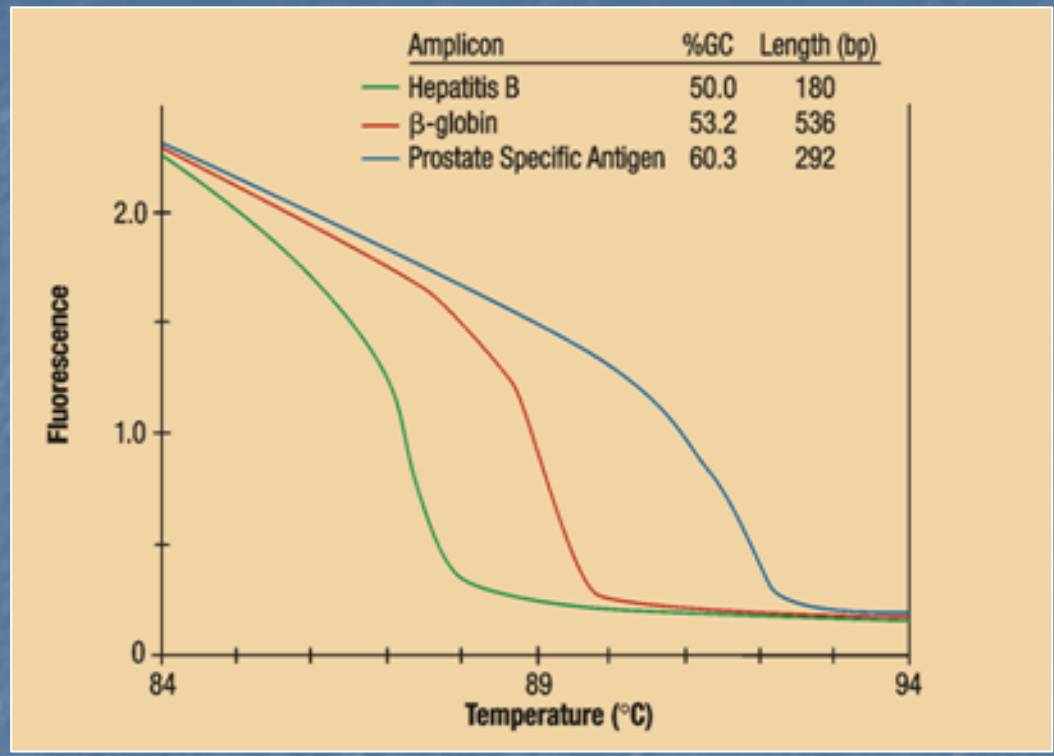
Back to theory …

Tm varies by:

- GC-content
- amplicon length

Tm is influenced by:

- Salt concentration
- $MgCl_2$ concentration
- dye concentration



Highest Stability **Lowest Stability**

G:C > A:T > G:G > G:T = G:A >T:T = A:A > T:C > A:C > C:C

BRCA genes are very challenging because of size and GC content!

Conclusions

- New software package v1.5:
  - Better performance
  - More user-friendly
- Normalization settings are not reproducible between labs
  - Use automatic software settings
- Specificity needs improvement → adjust sensitivity setting
- High sensitivity close to 99%...
- … however still 4 false negatives out of 296 tested samples
  - Current primer set not finalized yet for diagnostic purpose

Future work / actions

- Tackle false negative issues
  - Primer design
  - Secondary structures
  - GC content
  - Literature: touchdown PCR → increased specificity?
- Validation report
- Standard Operating Procedure (SOP) for practical use in diagnostic laboratories
- Poster at ESHG: P08.39
  - “Interlaboratory validation of High Resolution Melting (HRM) for BRCA1 and BRCA2 on the LightCycler® 480”

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