# *Er det arveligt eller hva´?*

## MLH1 promotor methyleringsanalyse og Mikrosatellit bestemmelse ved mave-tarm kræft

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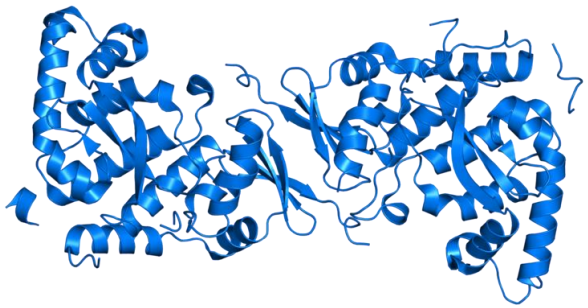


- Colorectal cancer (CRC) er den 3. mest almindelige kræfttype blandt mænd, og den 2. mest almindelige blandt kvinder

- Hovedparten af CRC er sporadiske, men 5% til 10% er arvelige med autosomale dominante mutationer.

- Den mest almindelige undertype af arvelig CRC er Lynch syndrome (hereditary nonpolyposis colon cancer, 3% til 5% af alle CRC)[1,2].

- Da arveligheden kan have betydning for øvrige familiemedlemmer er det væsentligt at undersøge for arvlighedsbiomarkører bl.a. for Lynch syndrome.

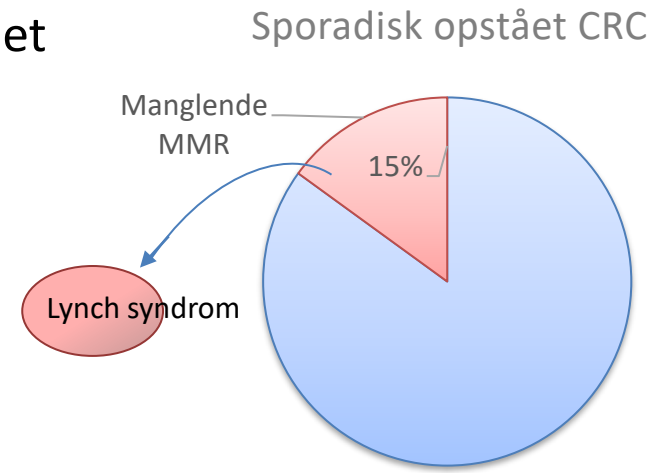
1. Lynch H.T., Snyder C.L., Shaw T.G., Heinen C.D., Hitchins M.P.: Milestones of Lynch syndrome: 1895-2015. Nat Rev Cancer 2015; 15: pp. 181-194.

2. Yamamoto H., Imai K.: Microsatellite instability: an update. Arch Toxicol 2015; 89: pp. 899-921.

# En ny måde at tænke MLH1 promotor methyleringsanalyse

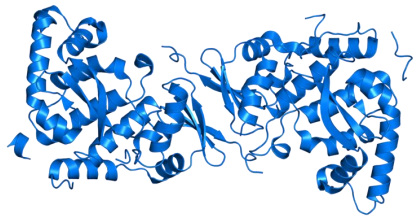
# Methyleringsanalyse ved kolorektalcancer

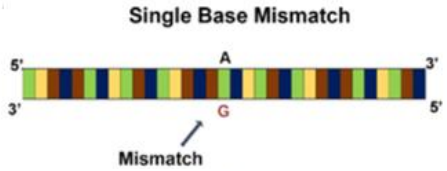
- Behandlingsindikator – arvelig komponent eller sporadisk opstået
- Sporadiske CRC: 15% har manglende mismatch repair proteiner (MMR)
- Et mindre antal af disse skyldes en arvelig gendefekt, Lynch Syndrom.




Er canceren så arvelig eller sporadisk?

*Det er her MLH1 og methylering kommer ind…*






# Hvad er *MLH1*?

*MLH1*-genet koder for MLH1, et DNA repair protein

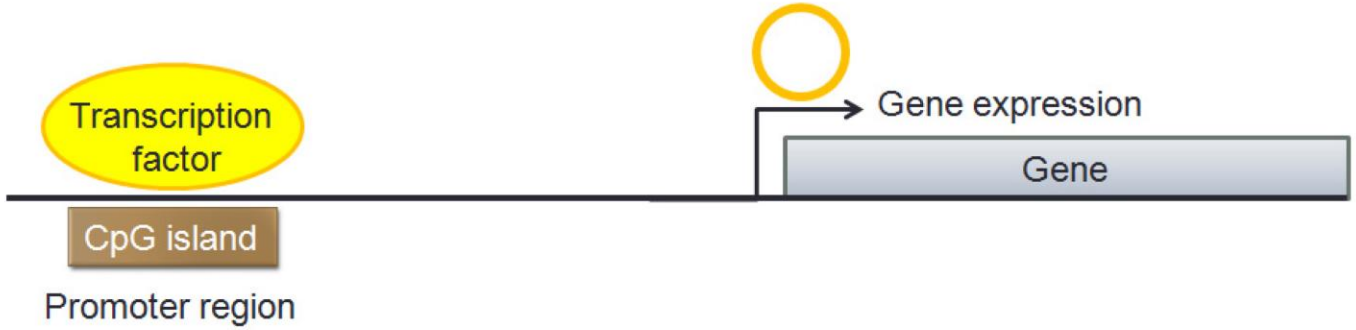
MLH1 er et af fire mismatch repair (MMR) proteiner der reparerer fejl efter replikation

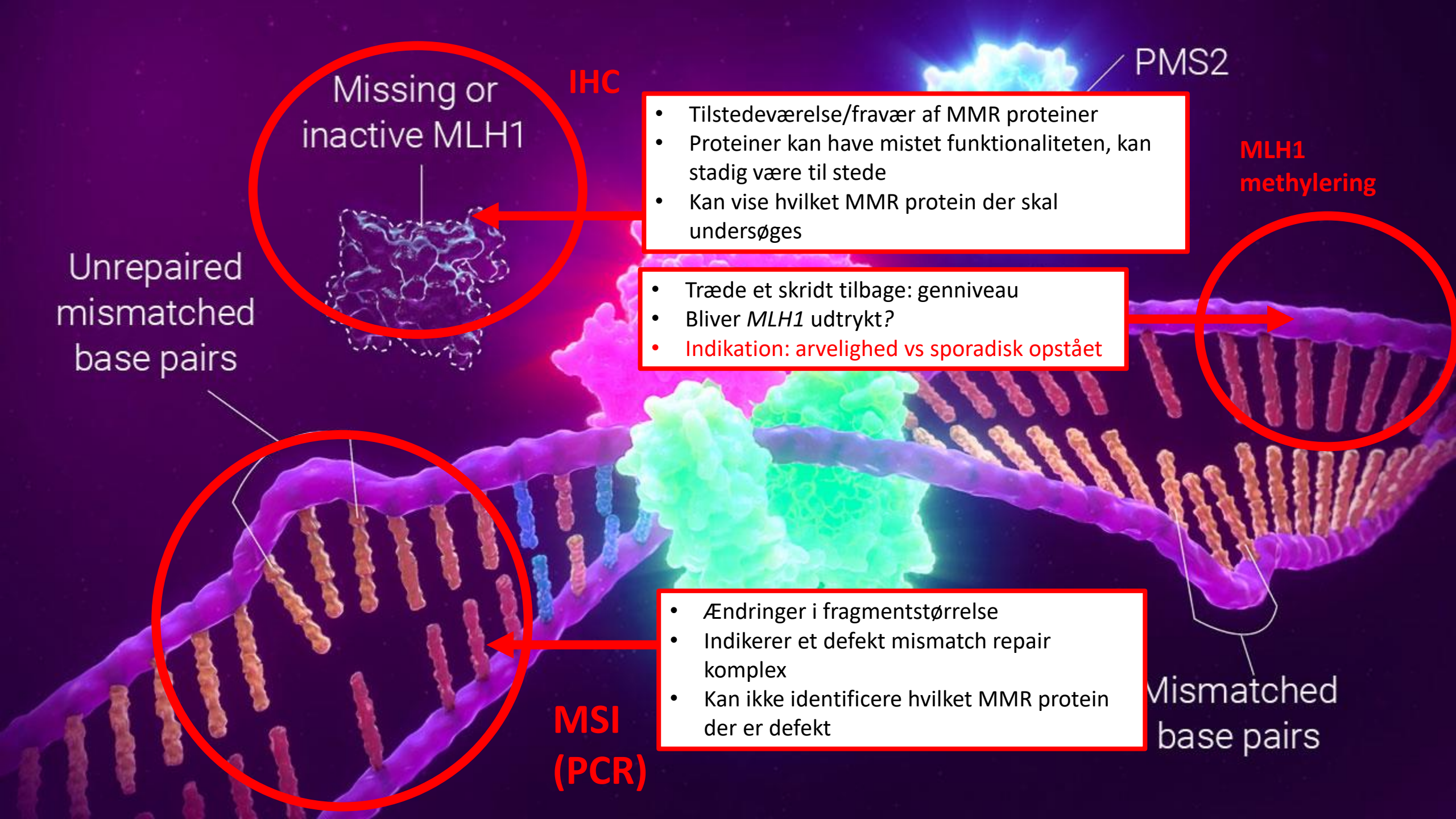
Ved methyleringsanalyse undersøges for methylering i promotoren til MLH1 genet

*Men hvad sker der når MLH1 genet er inaktiveret?*

# Hvad sker der når *MLH1* genet er inaktiveret?

**CpG islands** 1000-2000 bp med højt antal af CpG sites. Cytosin efterfulgt af guanin nukleotid.






**IHC**

- Tilstedeværelse/fravær af MMR proteiner
- Proteiner kan have mistet funktionaliteten, kan stadig være til stede
- Kan vise hvilket MMR protein der skal undersøges

**MLH1 methylering**

- Træde et skridt tilbage: genniveau
- Bliver *MLH1* udtrykt*?*
- Indikation: arvelighed vs sporadisk opstået

**MSI (PCR)**

- Ændringer i fragmentstørrelse
- Indikerer et defekt mismatch repair komplex
- Kan ikke identificere hvilket MMR protein der er defekt

# Workflow i arvelighed vs sporadisk opstået-problematikken

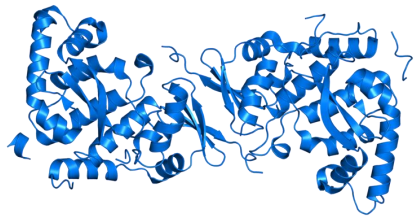
## Indikatorer

**Arvelig (Lynch syndrom)**

- Manglende MMR proteiner
- BRAF WT
- Methylering negativ

**Sporadisk opstået**

- Manglende MMR proteiner
- BRAF mut
- Methylering positiv

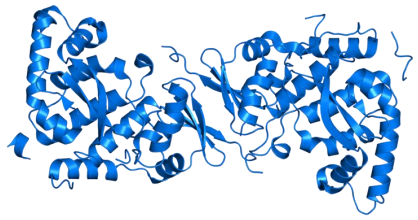


# Eksempler på analysemetoder

- SALSA MS-MLPA (MRC Holland): probebaseret + fragmentanalyse
- Pyrosekventering
- EpiMelt (MethylDetect): bisulfitkonvertering + PCR(HRM)
- *Next Generation Sequencing*

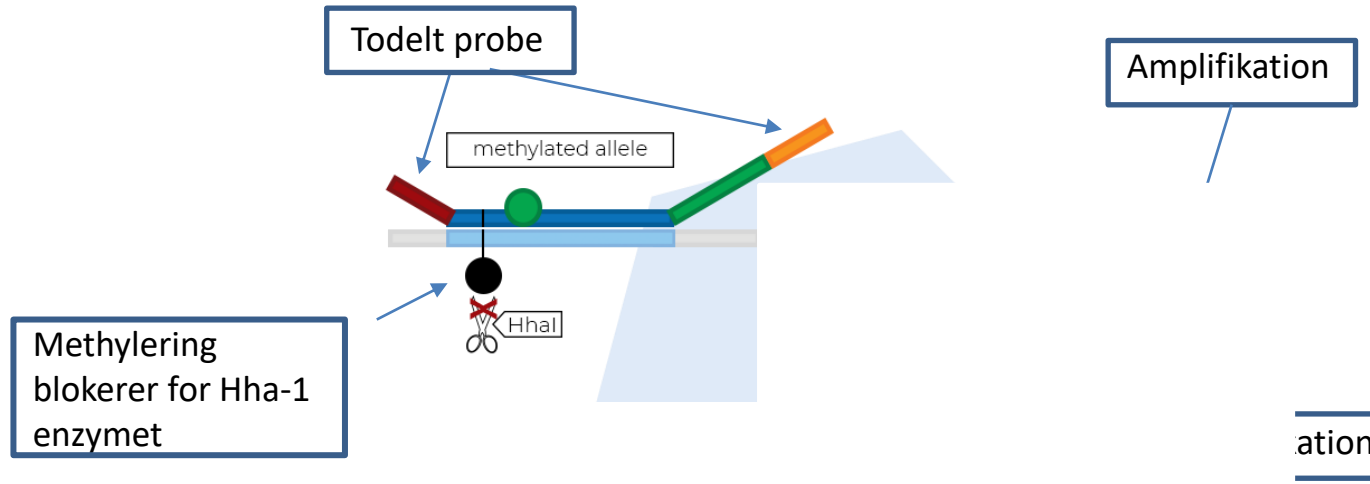
# Hvad en analyse skal leve op til ?

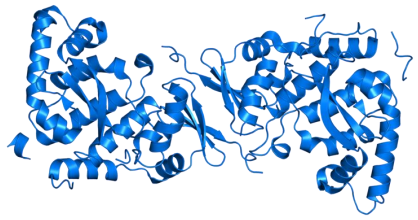
- Time-to-result
- Valideret
- Let at udføre
- Stabil
- Prisvenlig



# SALSA MS-MLPA

- Probebaseret
- Datanalyse: fragmentanalyse med kapillærelektroforese
- Input: 50-250 ng DNA
- Kræver ikke bisulfitkonvertering
- Tid: ~1 time hands on, 19 timer med inkubation = ca. 2 dage
- Fragmentanalyse tid: +/- 30 min (ifølge firma)
- 22 Hha-1 sites i 7 forskellige gener (MGMT, MLH1, MLH3, PMS2, MSH2, MSH3, MSH6)
- Tumorkerneindhold på minimum 20% anbefales



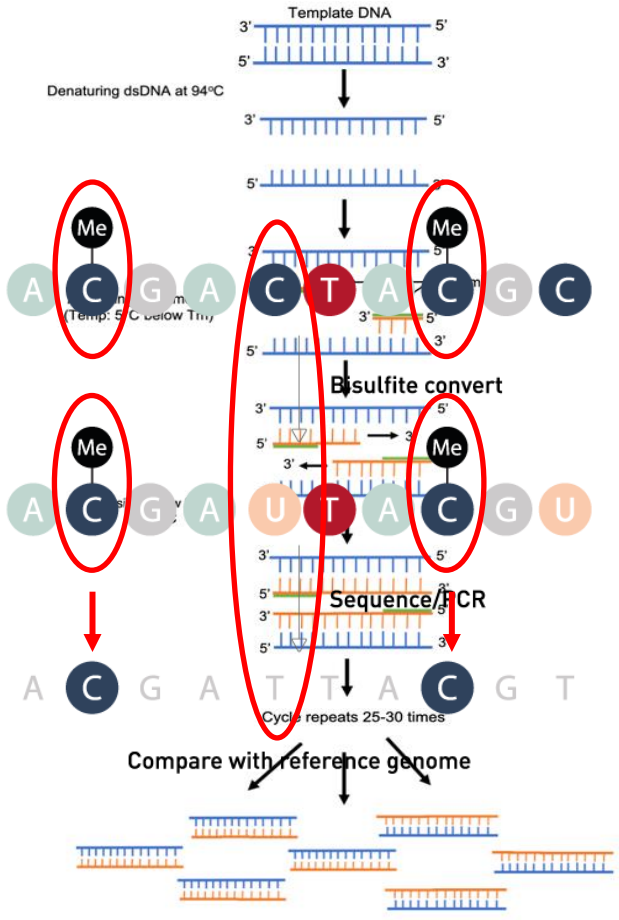



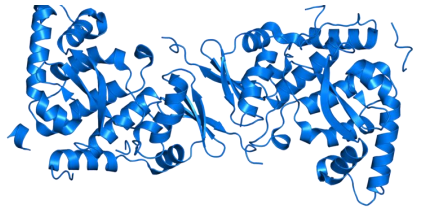
# EpiMelt

## Workflow

Ekstraktion → bisulfitkonvertering→PCR→dataanalyse (MS-HRM, High-Resolution Melting curves)

- Kræver bisulfitkonvertering
- Input: 500 pg-2 µg DNA til bisulfitkonvertering/10-100 ng bisulfitkonverteret DNA
- Tid: ~1 time hands on, ~6 timer med inkubation = ca. 1 dag
- Ét primer set til både methyleret og ikke-methylerede templates
- Ét gen (*MLH1*)
- Tumorkerneindhold anbefales: ingen anbefalinger. Har testet ned til 10%.



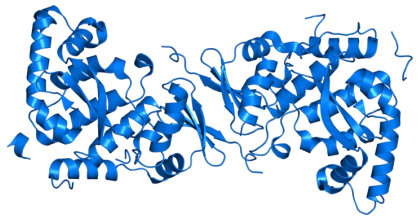
# EpiMelt MLH1 assay

**Hvordan sikrer vi kvaliteten?**

- **Ekstraktion** – ”garbage in, garbage out”

- **Bisulfitkonvertering**
  - Bland reagenser/prøve
  - Inkubationstid
  - Ratio: for meget DNA → kovertering ikke komplet (over-conversion/under-conversion)
  - Single-stranded: opbevaring i kort tid

- **PCR**
  - Optimal annealing temp
  - Optimal antal cycles
  - Sensitive reagenser



# EpiMelt MLH1 assay – opsætning i rutinen

**Prøver**

- 133 prøver inkluderet i valideringen
- 109 prøver analyseret på både RH og HVH
- Resektater og biopsier
- 14 med normal MMR

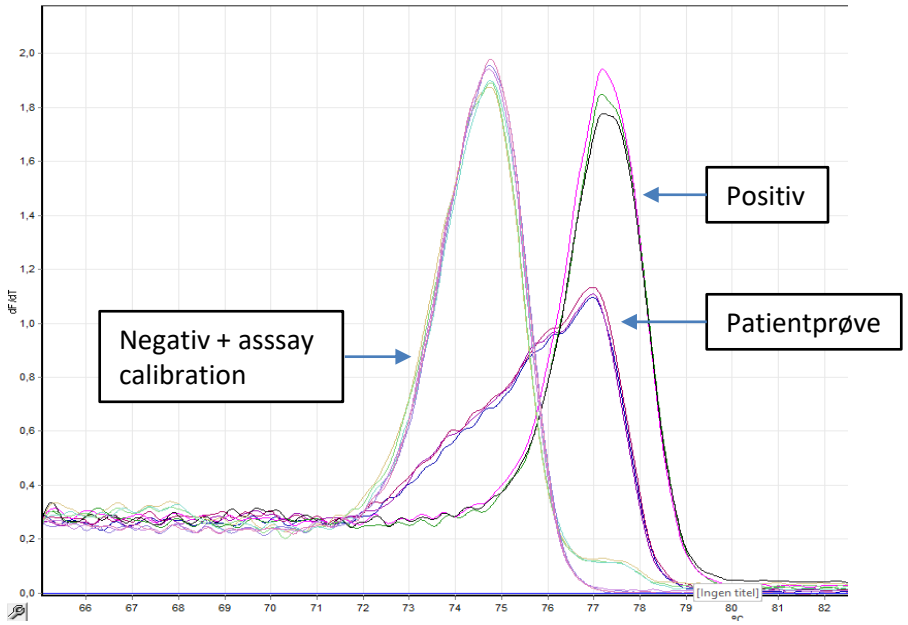
**Tre ekstraktionsmetoder benyttet**

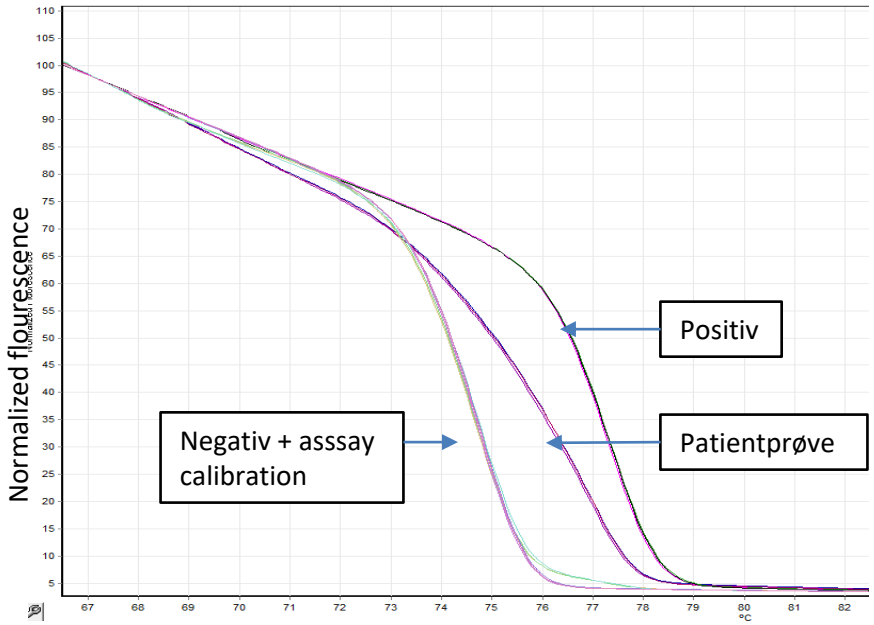
- Oprensning, QIAmp
- Oprensning, QIAamp med deparaffineringsopløsning og proteinase K-behandling over nat
- Oprensning med Promega Maxwell

**Annealingstest**

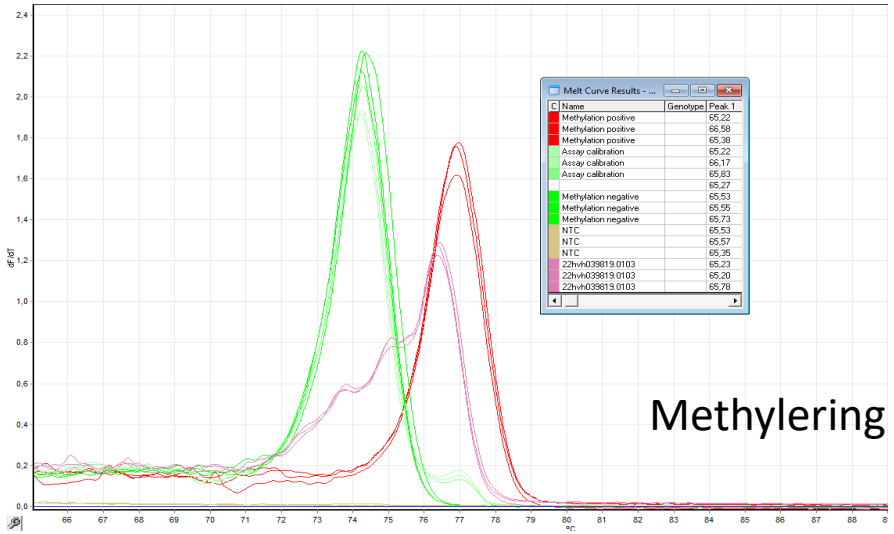
- Samme ekstraktion
- Optimering af PCR

# EpiMelt MLH1 assay – opsætning i rutinen

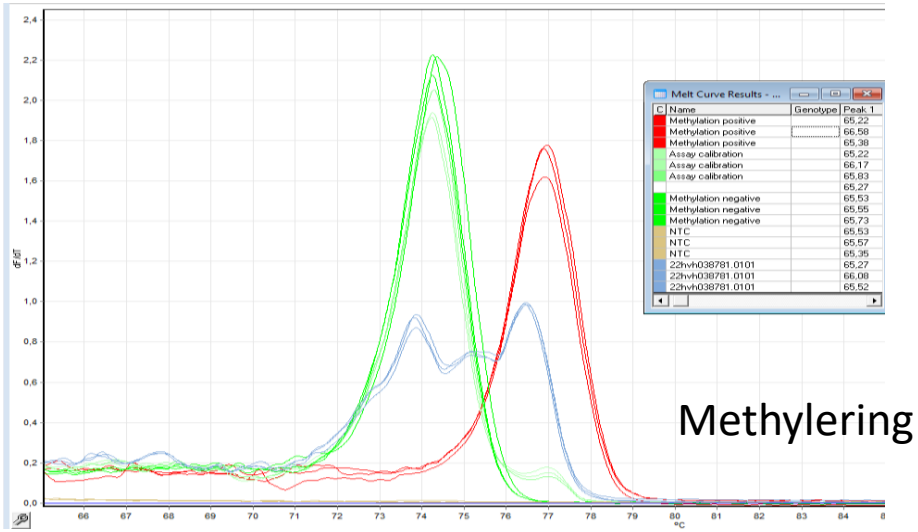


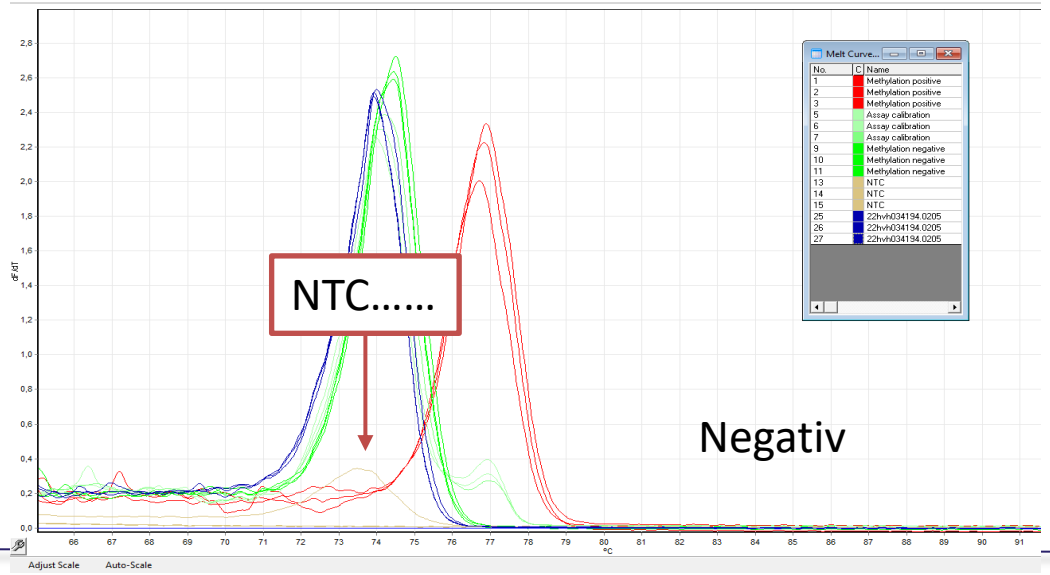
# Eksempler



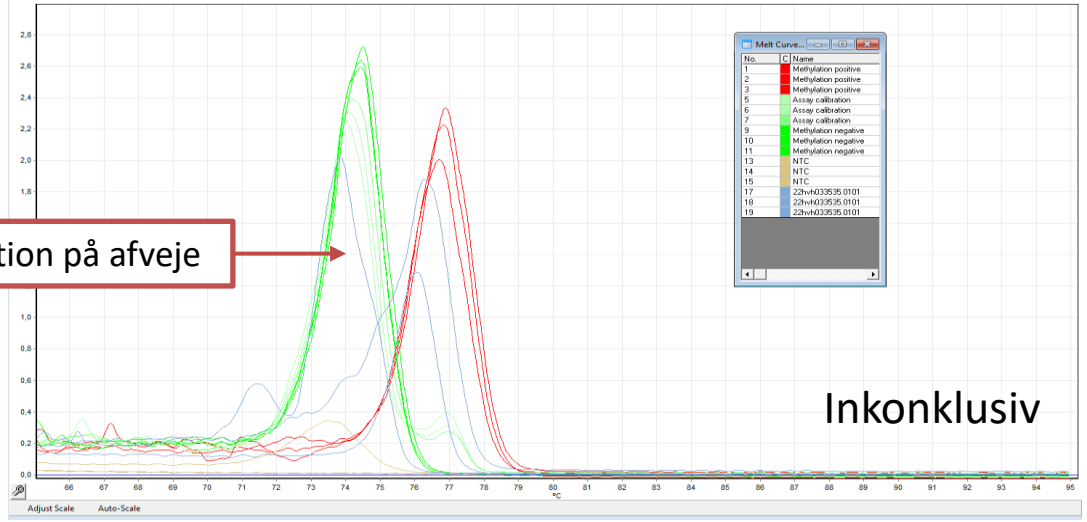

Methylering påvist




Methylering påvist



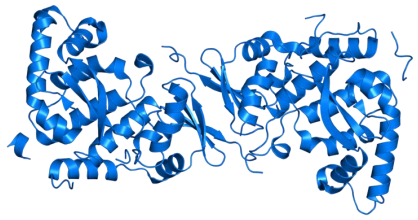

Negativ




Inkonklusiv

# Validering af
# EpiMelt MLH1 assay

*Er analysen reproducérbar og finder den samme cases som MLPA analysen?*

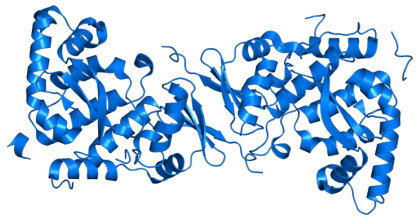


# Reproducérbarhed og konkordans

## Intern reproducérbarhed

| | | EpiMelt MLH1 | | | |
|---|---|---|---|---|---|
| | | Methyleret | Ikke methyleret | Invalid | Totalt |
| EpiMelt MLH1 | Methyleret | 15 | 0 | 0 | 15 |
| | Ikke methyleret | 1 | 2 | 0 | 3 |
| | Invalid | 0 | 0 | 0 | 0 |
| | Totalt | 16 | 2 | 0 | 18 |

Konkordans=94.4%
Kappa=0.77 (Substantial agreement)



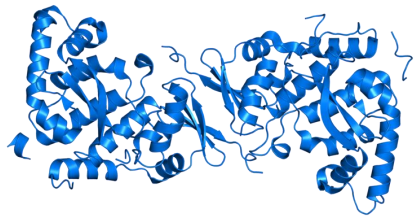
# Reproducérbarhed og konkordans

## Intern reproducérbarhed

| | | EpiMelt MLH1 | | | |
|---|---|---|---|---|---|
| | | Methyleret | Ikke methyleret | Invalid | Totalt |
| EpiMelt MLH1 | Methyleret | 15 | 0 | 0 | 15 |
| | Ikke methyleret | 1 | 2 | 0 | 3 |
| | Invalid | 0 | 0 | 0 | 0 |
| | Totalt | 16 | 2 | 0 | 18 |

* Skyldes meget lav koncentration ved anden kørsel (<0,5 ng/ul)

Konkordans=94.4%
Kappa=0.77 (Substantial agreement)



# Reproducérbarhed og konkordans

## Intern reproducérbarhed

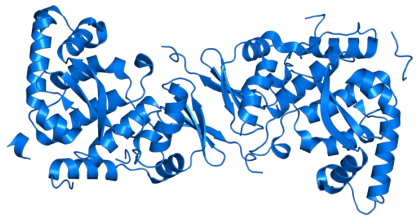
| | | EpiMelt MLH1 | | | |
|---|---|---|---|---|---|
| | | Methyleret | Ikke methyleret | Invalid | Totalt |
| EpiMelt MLH1 | Methyleret | 15 | 0 | 0 | 15 |
| | Ikke methyleret | 1 | 2 | 0 | 3 |
| | Invalid | 0 | 0 | 0 | 0 |
| | Totalt | 16 | 2 | 0 | 18 |

Konkordans=94.4%
Kappa=0.77 (Substantial agreement)

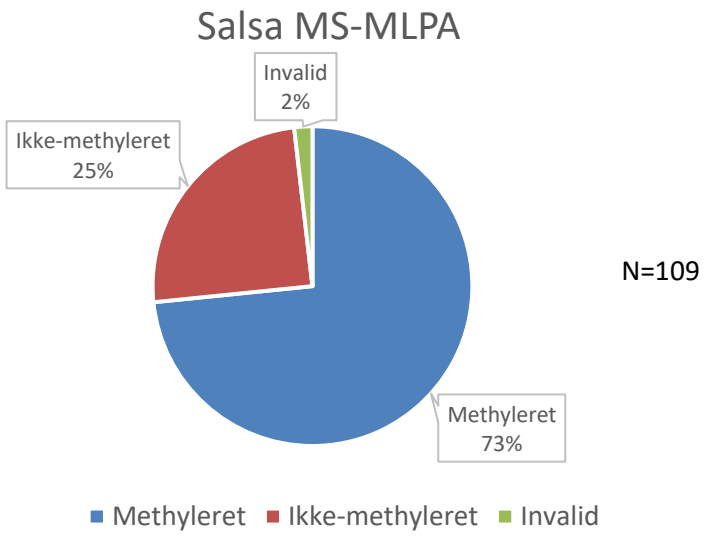
## Sammenligning af SALSA MS-MLPA og EpiMelt. N=109

| | | EpiMelt MLH1 | | | |
|---|---|---|---|---|---|
| | | Methyleret | Ikke methyleret | Invalid | Totalt |
| SALSA MS-MLPA | Methyleret | 80 | 0 | 0 | 80 |
| | Ikke methyleret | 10 | 17 | 0 | 27 |
| | Invalid | 1 | 1 | 0 | 2 |
| | Totalt | 91 | 18 | 0 | 109 |

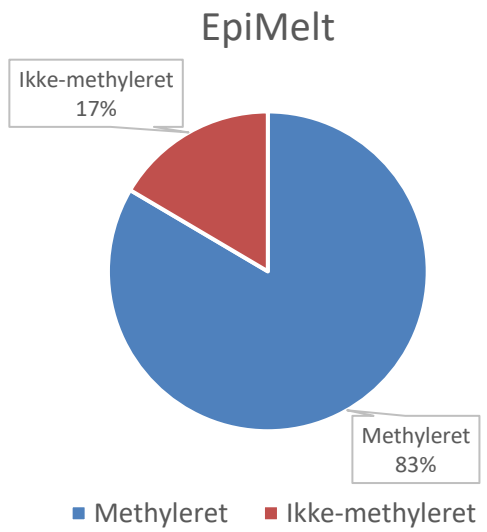
Konkordans=89%
Kappa=0.68 (Substantial agreement)



# Konkordans

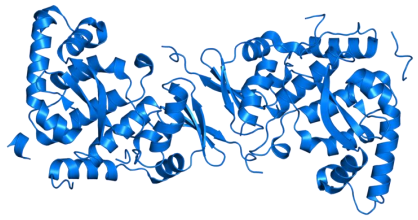



N=109




N=109

13.75% flere methyleringspositive



# Vores erfaringer med EpiMelt

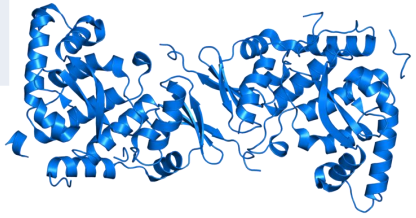
Sites/antal gener er forskellig fra assay til assay, dette kan, i nogle tilfælde, ændre methyleringsstatus

Jo bedre kvalitet og koncentration, jo bedre resultat, selvfølgelig…

Er færre gener/sites (22) nok? SALSA (7) vs EpiMelt (1)

Pris/time-to-result/labtidsforbrug ?

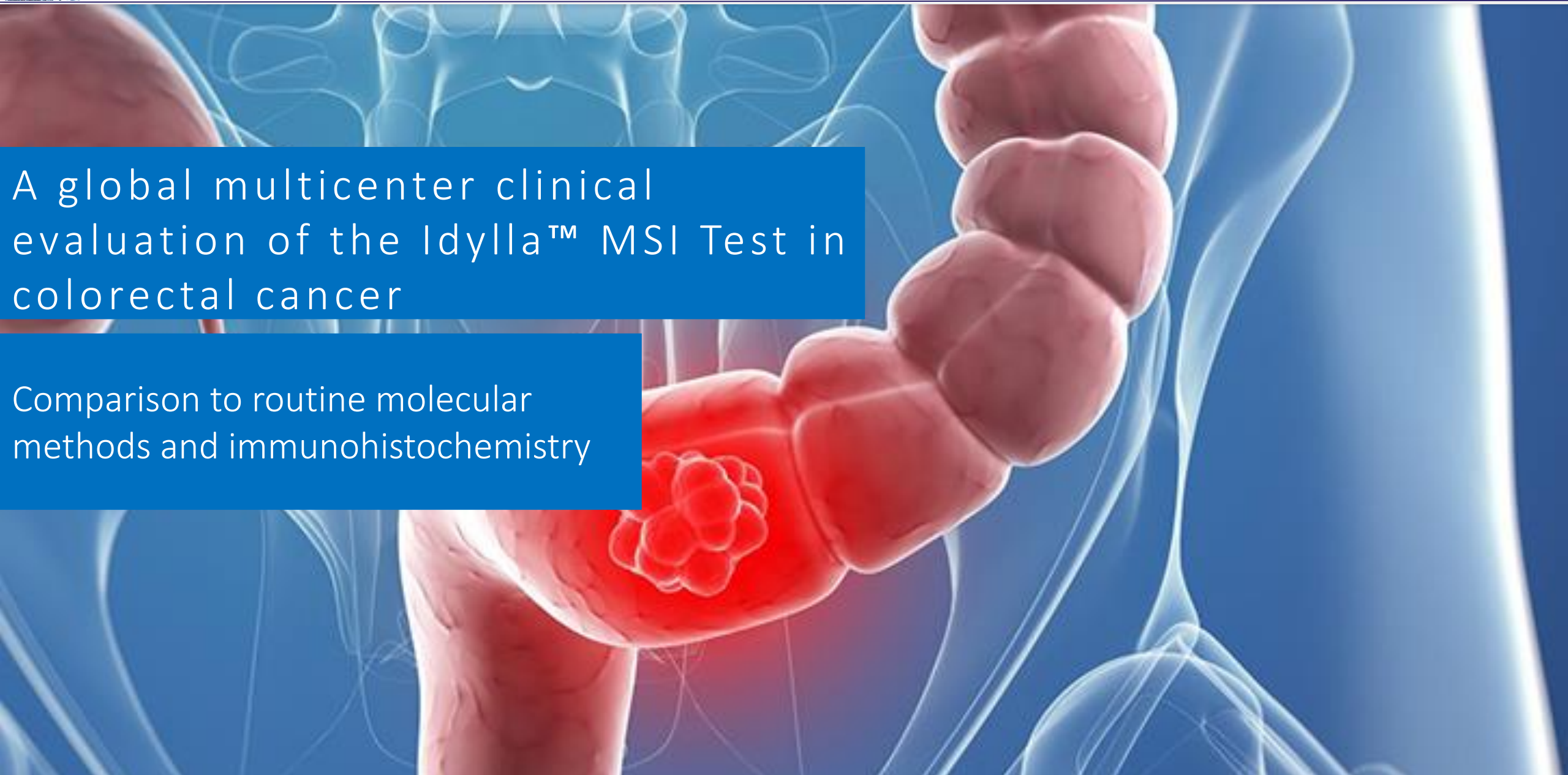
| | Pris | Tid |
|---|---|---|
| SALSA MS-MLPA | Ca. 101 kr. pr prøve | ~20,5 timer (1 times hands on) |
| EpiMelt | Ca. 7,5 kr. pr rxn<br>Køres i triplikater = 22,5 kr. pr. prøve | ~6 timer (1 times hands on) |



Molecular Pathology
Department of Pathology
Hvidovre Hospital

# A global multicenter clinical evaluation of the Idylla™ MSI Test in colorectal cancer

## Comparison to routine molecular methods and immunohistochemistry



## WHAT IS MICROSATELLITE INSTABILITY

- DNA polymerases makes errors in incorporating the correct number of bases during replication of long repetitive sequences, such as microsatellites
- Caused by mutational inactivation of genes involved in DNA repair

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- The MMR system is a family of proteins the detect and correct DNA replication errors
- The diagnostically important MMR proteins are PMS2, MLH1, MSH2, PMS1, and MLH3, MSH6.
- Description of differential expression of all or subsets of these proteins are conducted on CRC using IHC

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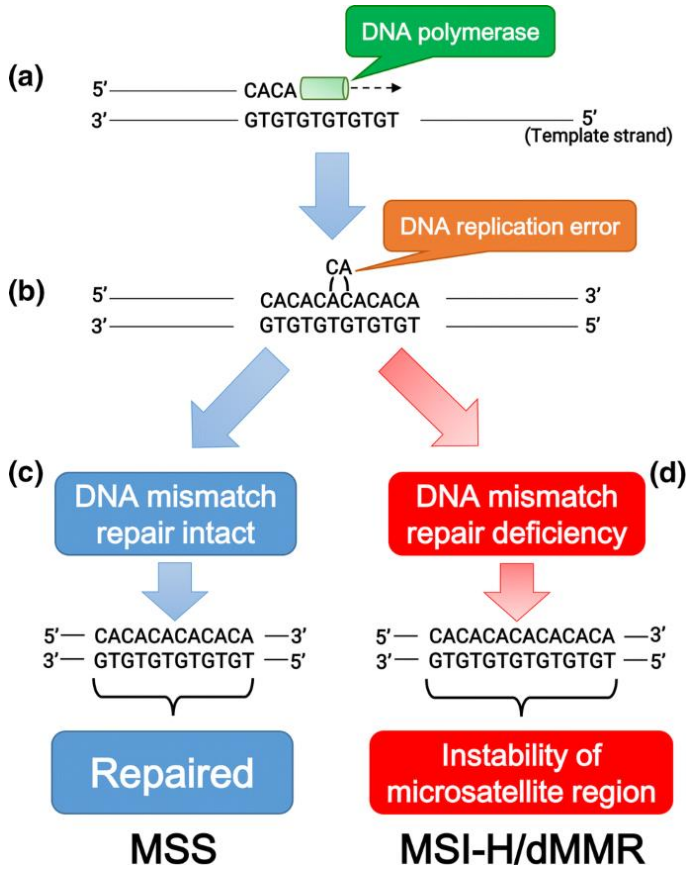
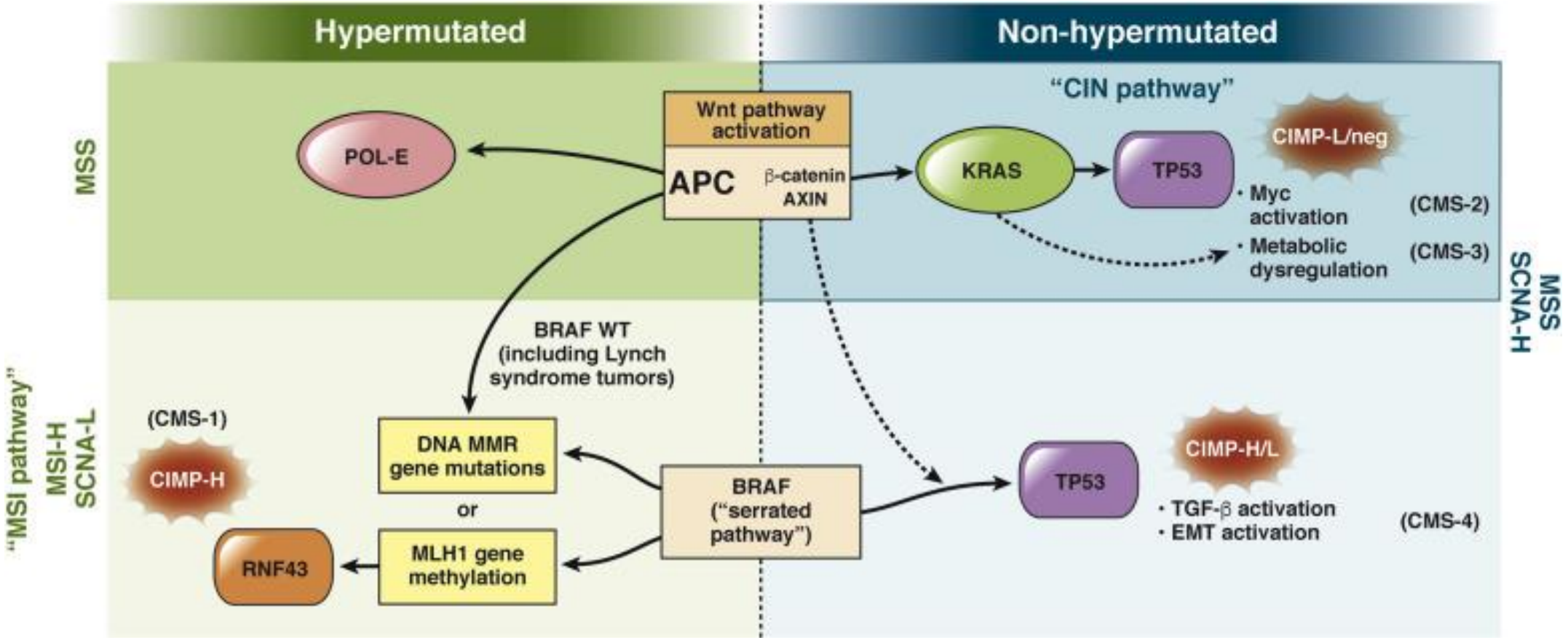
- Any mutations that occur in MMR genes will lead to deficient MMR repair resulting in an increase or decrease in microsatellite repeats

*THIS IS THE BASIS OF MSI-ANALYSIS.*




Eso, Y., Shimizu, T., Takeda, H. *et al.* Microsatellite instability and immune checkpoint inhibitors *J Gastroenterol* **55,** 15–26 (2020)

# MICROSATELLITE INSTABILITY IN COLORECTAL CARCINOMA




Long H. Nguyen, Ajay Goel, Daniel C. Chung
Gastroenterology. 2020 January ; 158(2): 291–302

## There are many putative MSI markers described in the literature
*A few definitions*

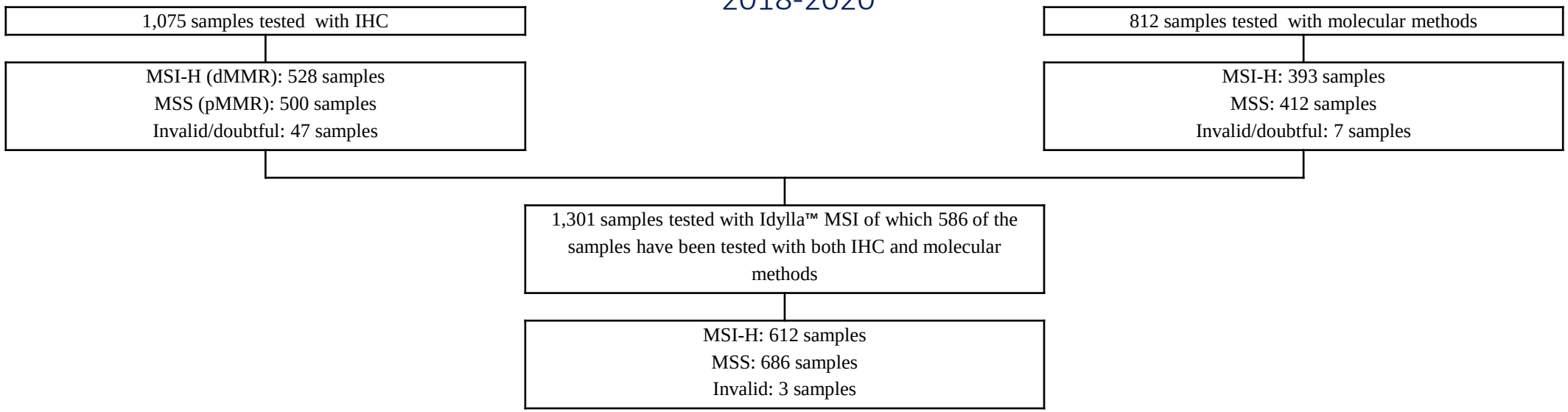
- The National Cancer Institute proposed the so-called Bethesda consensus-defined panel of microsatellites, which include two mononucleotide tracts (BAT-25 and BAT-26), and three dinucleotide repeats (D5S346, D2S123, and D17S250), with a sensitivity reported around 90%.
- CRC is classified as MSI-L when one of the five microsatellite markers show instability (deletions or insertions)
- CRC is classified as MSI-H when two or more of the five microsatellite markers show instability (deletions or insertions)
- CRC is categorized as microsatellite stable (MSS) if variability in microsatellite markers size compared to normal tissue is seen in no marker.
- MSI-L or MSI-H; lack of consensus on the clinical implication, data missing on tumor homo- or heterogeneousity

# The Idylla™ MSI Assay

| Marker | Locus |
|---|---|
| ACVR2A | 2q22.3-q23.1 |
| BTBD7 | 14q32.12 |
| DIDO1 | 20q13.33 |
| MRE11 | 11q21 |
| RYR3 | 15q13.3-q14 |
| SEC31A | 4q21.22 |
| SULF2 | 20q13.12 |

# THE STUDY

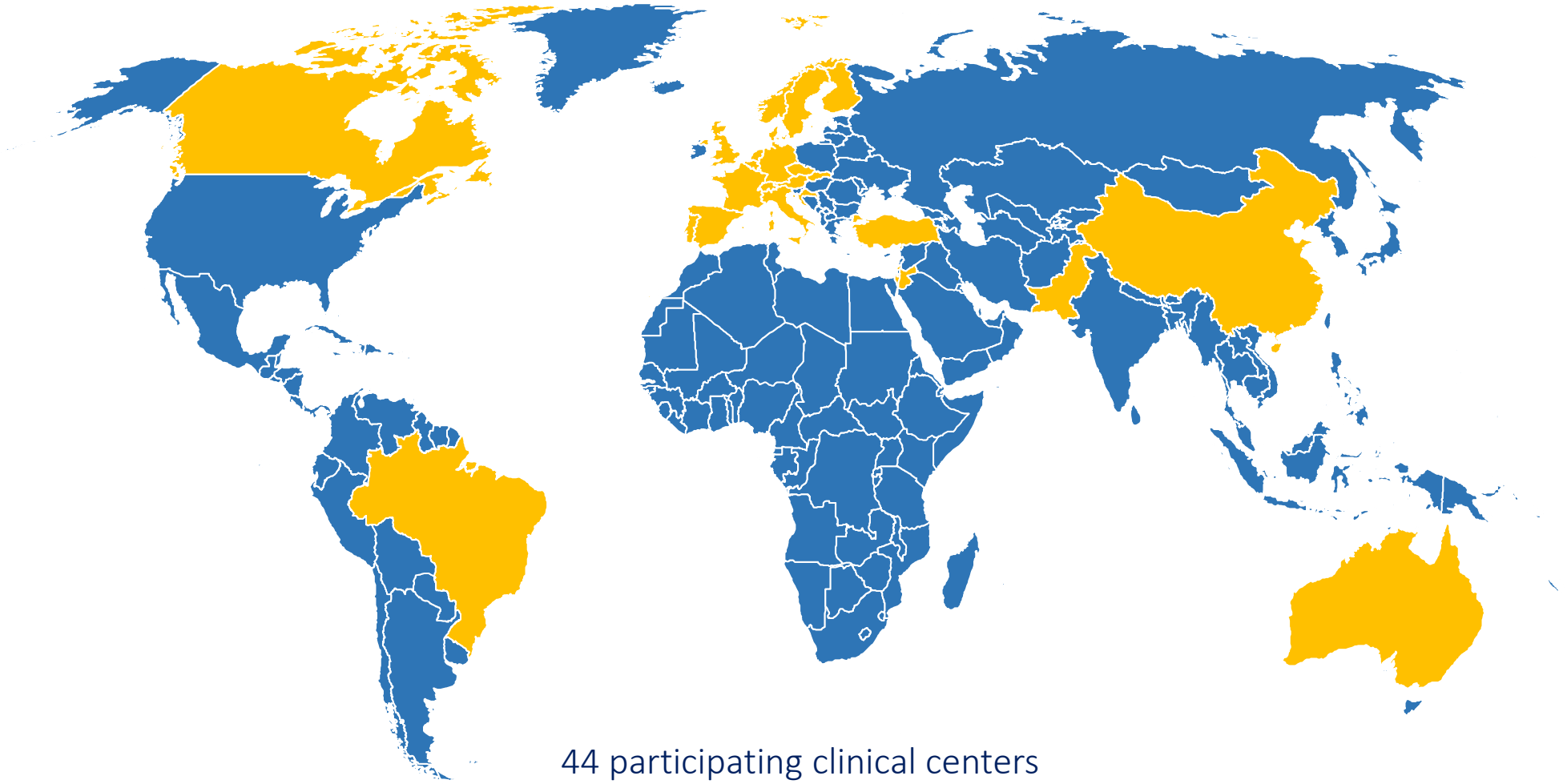
44 participating clinical centers
2018-2020




- 1,075 samples had been tested before with IHC
- 812 with molecular methods.

- Of the 812 samples tested with molecular methods, 101 had been tested with the original Bethesda panel, 525 with the revised Bethesda panel, and 186 against a range of other microsatellite biomarker panels.

# A WORLD WIDE STUDY



44 participating clinical centers

## Characteristics of study samples

| Tissue origin | # |
|---|---|
| Primary | 969 |
| Metastatic | 48 |
| NA | 284 |
| ∑ | 1301 |

| Slice thickness (µm) | # |
|---|---|
| 3 | 30* |
| 4 | 23* |
| 5 | 671 |
| 8 | 1 |
| 10 | 553 |
| NA | 23 |

| Number of slices | # |
|---|---|
| 1 | 918 |
| 2 | 164 |
| 3 | 120 |
| 4 | 52 |
| 5 | 19 |
| 6 | 3* |
| 7 | 1* |
| 8 | 1* |
| 11 | 1* |
| 12 | 1* |
| NA | 21 |

| % tumor cells (after macrodissection) | # |
|---|---|
| <10 | 4* |
| 10 - <20 | 20* |
| 20 - <30 | 76 |
| 30 - <40 | 121 |
| 40 - <50 | 131 |
| 50 - <60 | 157 |
| 60 - <70 | 165 |
| 70 - <80 | 145 |
| 80 - <90 | 113 |
| 90 - 100 | 84 |
| NA | 285 |

*In the study, the test material input variables were wide and reflected the entire spectrum of clinical diagnostic situations*

*Values not according to the specifications of the Idylla™ MSI assay instructions; however, for all these samples, Idylla™ MSI assay results were found concordant with results of previous routine reference methods

# MSS vs MSI CALLS STRATIFIED ON NUMBER OF MARKER CALLS

| MSI status | Number of samples | Number of mutant markers | Number of samples | % of MSI-H |
|---|---|---|---|---|
| MSS | 686 | 0 | 674 | NA |
| | | 1 | 12 | NA |
| MSI-H | 612 | 2 | 15 | 2.45 |
| | | 3 | 28 | 4.58 |
| | | 4 | 52 | 8.50 |
| | | 5 | 156 | 25.49 |
| | | 6 | 226 | 36.93 |
| | | 7 | 135 | 22.06 |
| Invalid | 3 | NA | NA | NA |
| Total | 1301 | | | |

93% MSI calls on ≥4 markers

Very few calls on MSI-L

# Idylla™ MSI calls per biomarker

| | ACVR2A | BTBD7 | DIDO1 | MRE11 | RYR3 | SEC31A | SULF2 |
|---|---|---|---|---|---|---|---|
| **Overall** | | | | | | | |
| Mutant | 579 | 514 | 576 | 505 | 411 | 373 | 457 |
| Wild type | 718 | 782 | 725 | 780 | 887 | 926 | 840 |
| Invalid | 4 | 5 | 0 | 16 | 3 | 2 | 4 |
| Total | 1,301 | 1,301 | 1,301 | 1,301 | 1,301 | 1,301 | 1,301 |
| **MSI-H samples** | | | | | | | |
| Mutant | 575 | 513 | 570 | 504 | 411 | 373 | 457 |
| Wild type | 37 | 99 | 42 | 107 | 201 | 239 | 154 |
| Invalid | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| Total | 612 | 612 | 612 | 612 | 612 | 612 | 612 |
| **MSS samples** | | | | | | | |
| Mutant | 4 | 1 | 6 | 1 | 0 | 0 | 0 |
| Wild type | 680 | 683 | 680 | 672 | 685 | 686 | 685 |
| Invalid | 2 | 2 | 0 | 13 | 1 | 0 | 1 |
| Total | 686 | 686 | 686 | 686 | 686 | 686 | 686 |
| **Invalid samples** | | | | | | | |
| Mutant | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Wild type | 1 | 0 | 3 | 1 | 1 | 1 | 1 |
| Invalid | 2 | 3 | 0 | 2 | 2 | 2 | 2 |
| Total | 3 | 3 | 3 | 3 | 3 | 3 | 3 |

# MSI-H vs. MSI-L

When MSI-H status is detected, generally loss of expression of the set MLH1/PMS2 or MSH2/MSH6 is observed.

However, the sole loss of one marker can take place in some cases of Lynch syndrome that do not display MSI-H.

**So what to do?**

In case of one MSI-L, conduct MMR-IHC until such time as the evidence is solidified

# Idylla™ MSI performance compared to IHC

| | | IHC | | | | |
|---|---|---|---|---|---|---|
| | | dMMR | pMMR | Invalid | Doubtful | Total |
| Idylla™ | MSI-H | 501 | 12 | 1 | 10 | 524 |
| | MSS | 25 | 487 | 6 | 30 | 548 |
| | Invalid | 2 | 1 | 0 | 0 | 3 |
| | Total | 528 | 500 | 7 | 40 | 1075 |
| Idylla™ Performance | Positive agreement | 501/526 = 95.24% (CI: 93.08–96.76%) | | | | |
| | Negative agreement | 487/499 = 97.59% (CI: 95.84–98.62%) | | | | |
| | Overall agreement | 988/1025 = 96.39% (CI: 95.06–97.37%) | | | | |

## COMPARATOR

IHC analysis of the expression of the marker genes *MLH1*, *MSH2*, MSH6, and *PMS2* in FFPE tissue material was performed using routine standard protocols and equipment, including commercial antibodies from Ventana (Roche Diagnostics, Rotkreuz, Switzerland) and Dako (Agilent, Santa Clara, CA).

# Idylla™ MSI performance compared to similar molecular MSI methods

| | | Molecular methods | | | | |
|---|---|---|---|---|---|---|
| | | MSI-H | MSS | Invalid | Doubtful | Total |
| **Idylla™** | MSI-H | 381 | 4 | 0 | 2 | 387 |
| | MSS | 12 | 408 | 1 | 2 | 423 |
| | Invalid | 0 | 0 | 2 | 0 | 2 |
| | Total | 393 | 412 | 3 | 4 | 812 |
| **Idylla™ Performance** | Positive agreement | 381/393 = 96.95% (CI: 94.74–98.24%) | | | | |
| | Negative agreement | 408/412 = 99.03% (CI: 97.53–99.62%) | | | | |
| | Overall agreement | 789/805 = 98.01% (CI: 96.80–98.77%) | | | | |

COMPARATOR
Promega MSI Analysis System v1.2
PCR analysis was performed on customized molecular MSI panels

## *Clinical Performance of the Idylla™ MSI Test for a Rapid Assessment of the DNA Microsatellite Status in Human Colorectal Cancer*

Zwaenepoel et al, Journal of Molecular Diagnostics, The, 2020-03-01,
Volume 22, Issue 3, Pages 386-395,

| **Idylla** | **Promega** | | |
|---|---|---|---|
| | **Valid** | **Invalid** | **Total** |
| **Valid** | 323 | 5 | 328 |
| **Invalid** | 0 | 2 | 2 |
| **Total** | 323 | 7 | 33 |

## Conclusion:

The OPA was 99.7% (95% CI, 98.3%–100%).
The PPA was 98.7% (95% CI, 92.9%–99.8%), and the NPA was 100% (95% CI, 98.5%–100%).

In conclusion, the Idylla™ MSI Test has been shown to be extremely powerful in identifying the microsatellite status in human CRC samples

# MSI as diagnostic tool in other cancers, and for treatment decisions

1

MSI is also observed in about 15% of gastric and endometrial cancers, and at lower frequencies in several other cancers[1].

2

Approval for anti-PD1 immune-checkpoint inhibition has been granted for all patients with solid tumors containing MSI-H or being MMR deficient[2,3].

*1 Bonneville et al.* Landscape of microsatellite instability across 39 cancer types. JCO Precision Oncol 2017; 2017.
*2 Ryan et al.* The current value of determining the mismatch repair status of colorectal cancer: a rationale for routine testing. Crit Rev Oncol Hematol 2017; 116: pp. 38-57.
*3 Abida et al.* Analysis of the prevalence of microsatellite instability in prostate cancer and response to immune checkpoint blockade. JAMA Oncol 2019; 5: pp. 471-478.

# CONCLUSION

The Idylla™ MSI Assay showed high concordances with IHC and molecular testing

Extremely simple workflow and short turnaround time

No Pathologist review required

Required limited amount of tumor tissue, working over a wide range of material amounts

Requires no matched normal tissue as for IHC  or Promega MSI test

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