Next Generation Sequencing voor moleculaire diagnostiek

EQA voor NGS?

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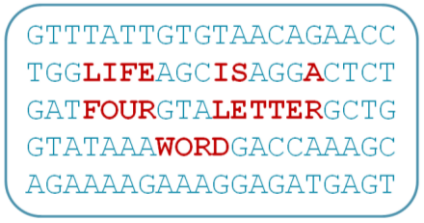
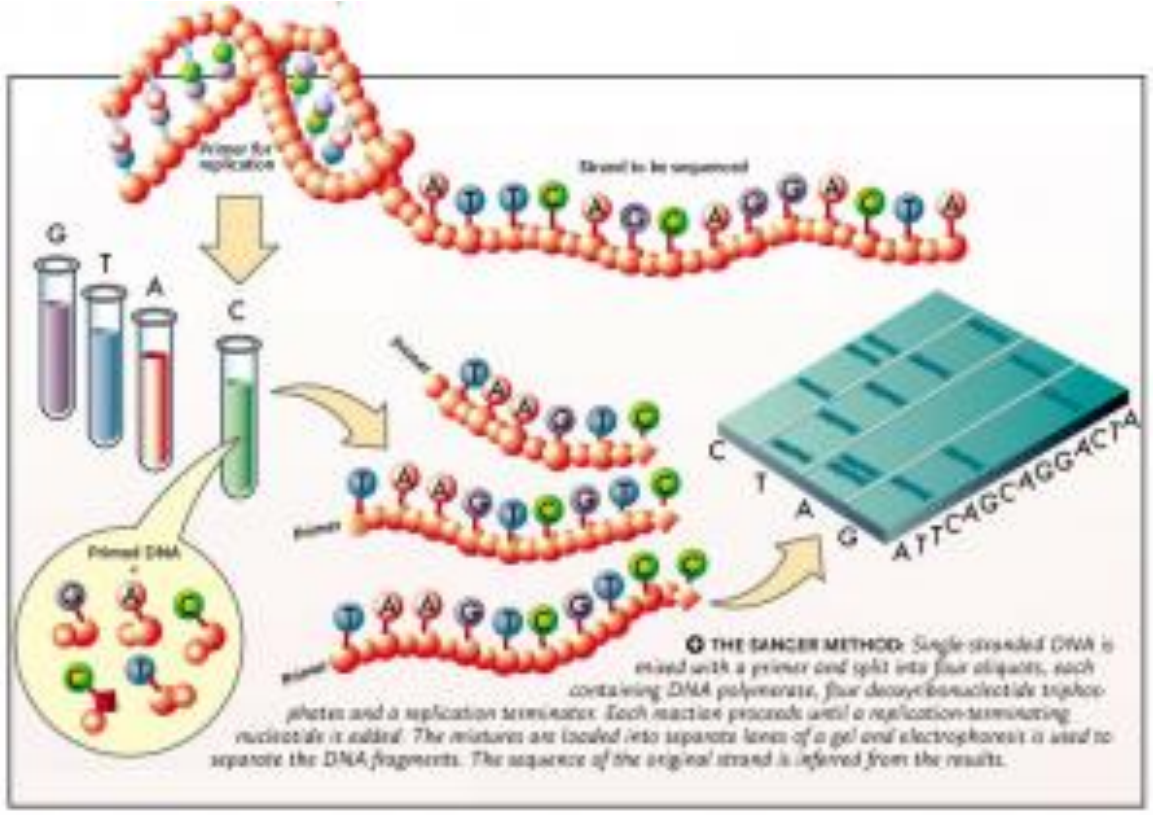
Laboratory Hematology



Radboudumc

Sequentie analyse

‘Old school’ Sanger sequentie analyse (Sanger, 1977)
Sequencing-by-synthesis

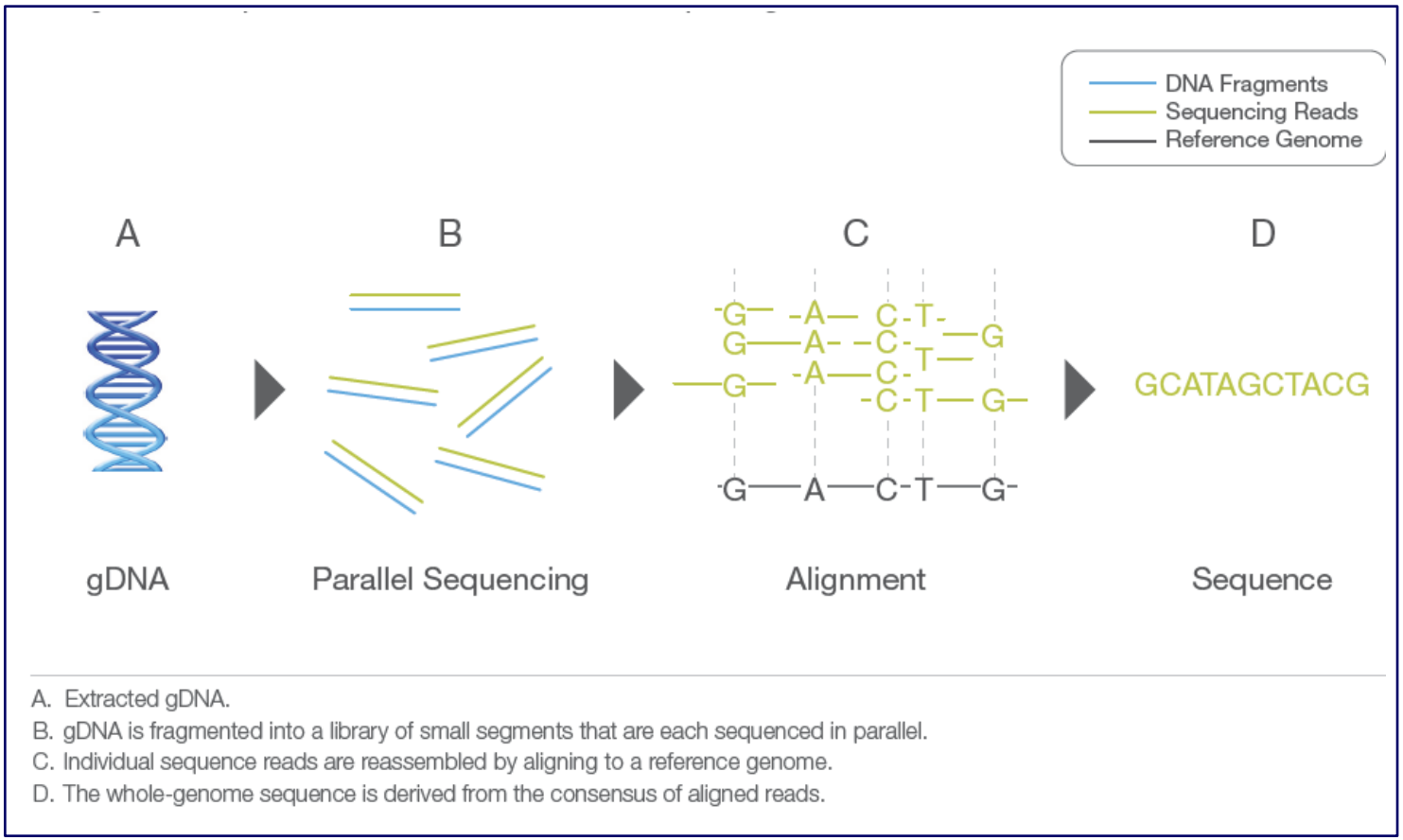


Sequencing van één DNA fragment per keer, ca. 800-1000 bp

Next Generation Sequencing (NGS)

Massively parallel Sequencing (2000)

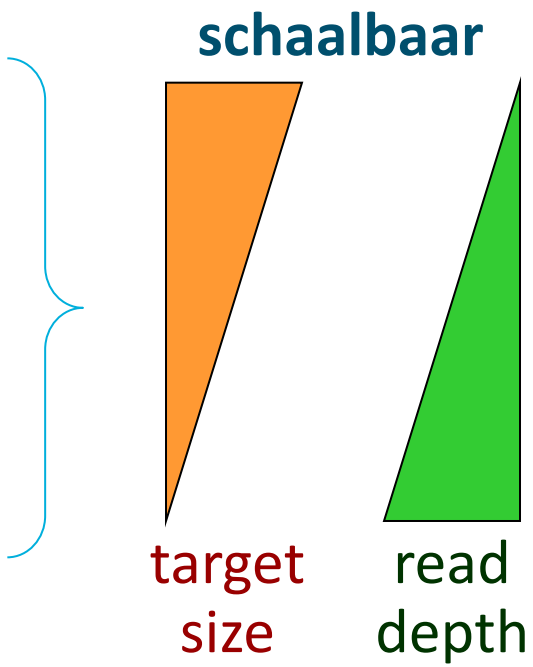
Meeste methoden gebruiken ‘Sequencing-by-synthesis’ principe, maar dan op nanoschaal in miljoenen parallele sequencing reacties



A. Extracted gDNA.
B. gDNA is fragmented into a library of small segments that are each sequenced in parallel.
C. Individual sequence reads are reassembled by aligning to a reference genome.
D. The whole-genome sequence is derived from the consensus of aligned reads.

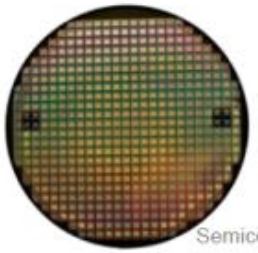
Next Generation Sequencing (NGS)

- Whole-genome sequencing (3 x 10^9 bp)
- Whole-exome sequencing (30 x 10^6 bp)
- Targeted / amplicon-based sequencing
- Deep (targeted) sequencing

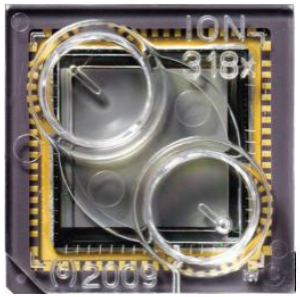


Ion Torrent - semiconductor sequencing

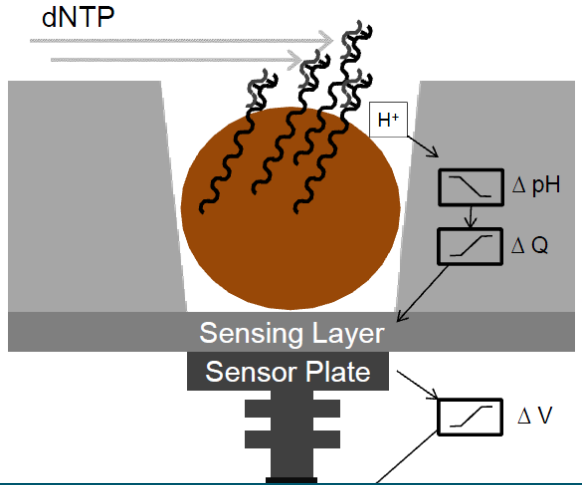
ion torrent
by life technologies™

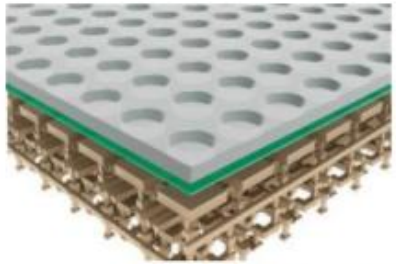


Wafer
Semiconductor Manufacturing



Chip
Semiconductor Packaging

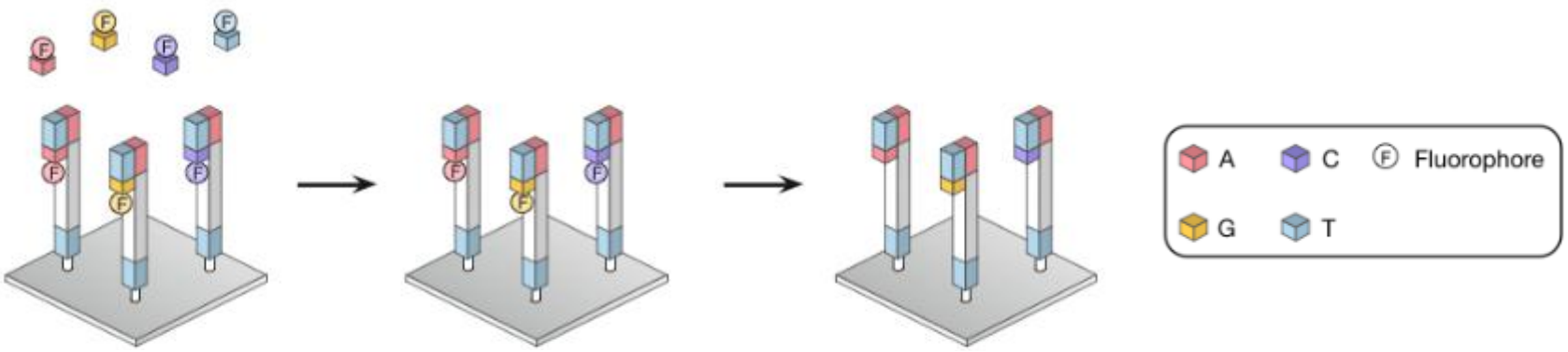




Millions of Sensors
Semiconductor Design

Nucleotiden zijn niet gelabeld
Per cyclus wordt maar 1 type base ingebouwd (A, C, T of G)
Meerdere van dezelfde base kunnen in 1 cyclus worden ingebouwd
→ homopolymere regio's!
Signaal detectie: pH sensor per individuele microwell registreert inbouw
Basen tijdens sequencing-by-synthesis

Illumina - reversible terminator sequencing



Primers are extended by one nucleotide, with dye and terminator

Wash away excess nucleotides imaging

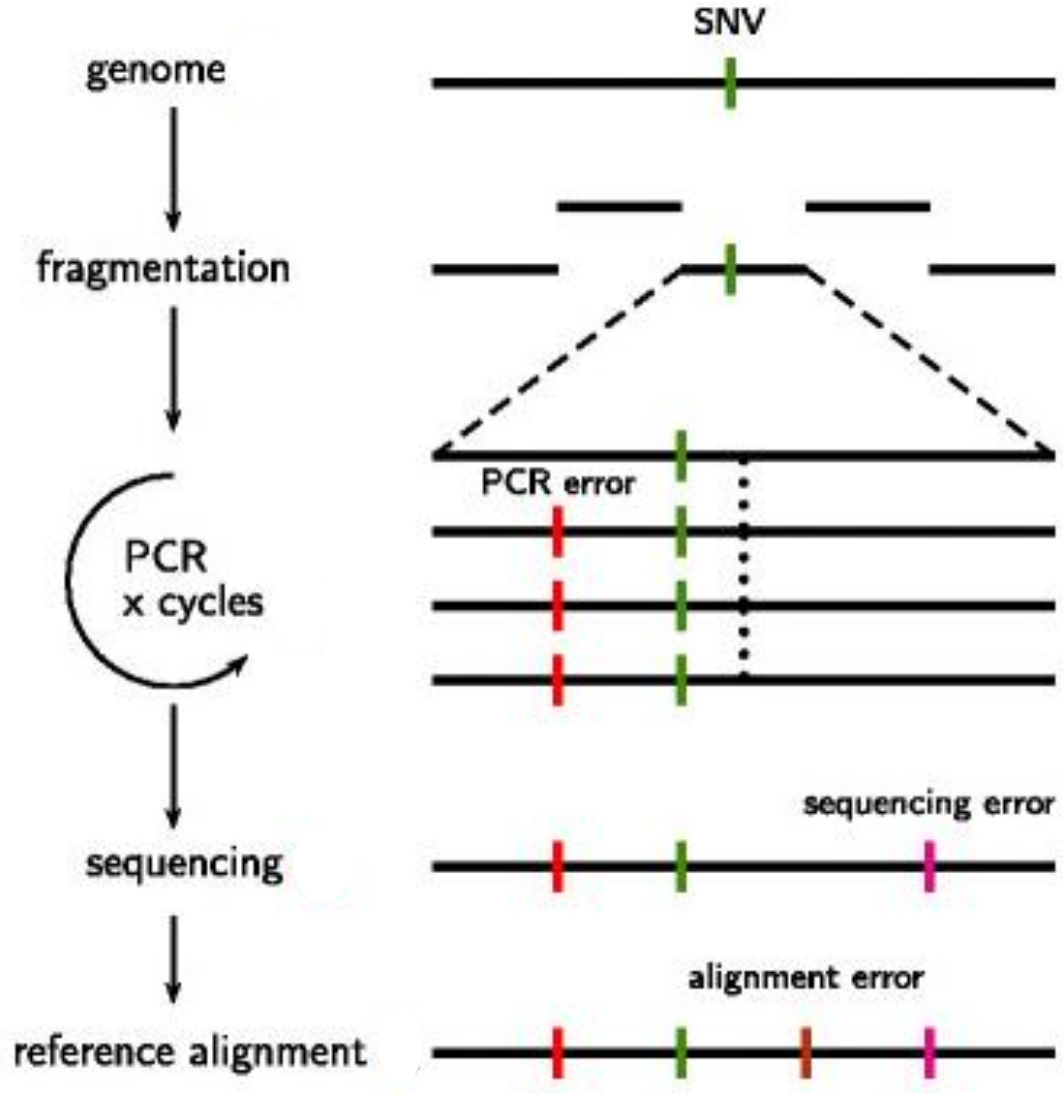
Dye and terminator are removed

Nucleotiden zijn gelabeld met fluorescente marker en reversibele terminator

Per cyclus wordt slechts 1 nucleotide ingebouwd (terminator)

Signaal detectie: fotodetectie van fluorescent signaal per cluster

Errors in NGS



Errors kunnen leiden tot fout-positieve variant calls

Random errors

Willekeurige fouten

Meestal op laag niveau

Meestal maar in één monster in een run

Niet-random errors

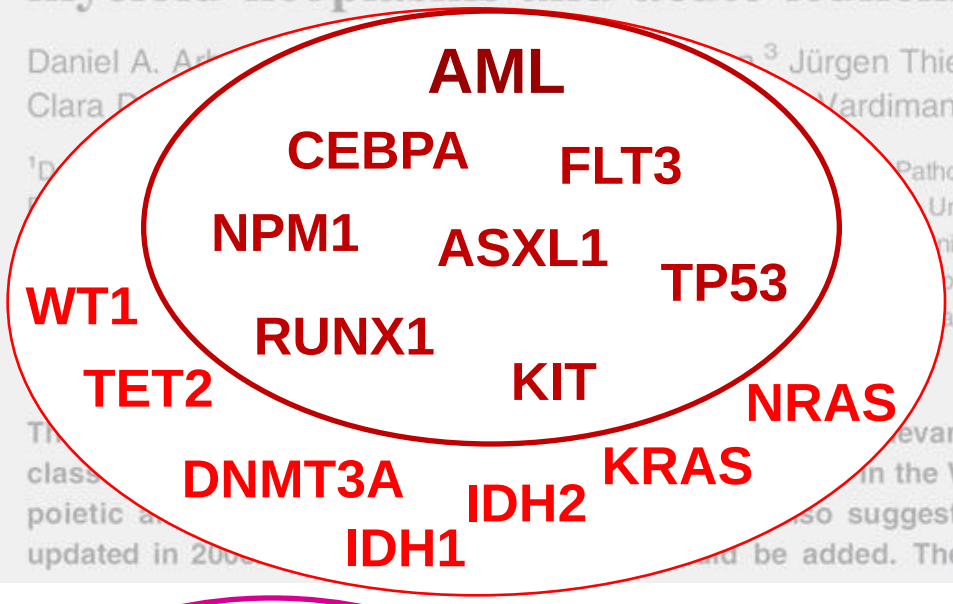
Kunnen op hoger niveau voorkomen

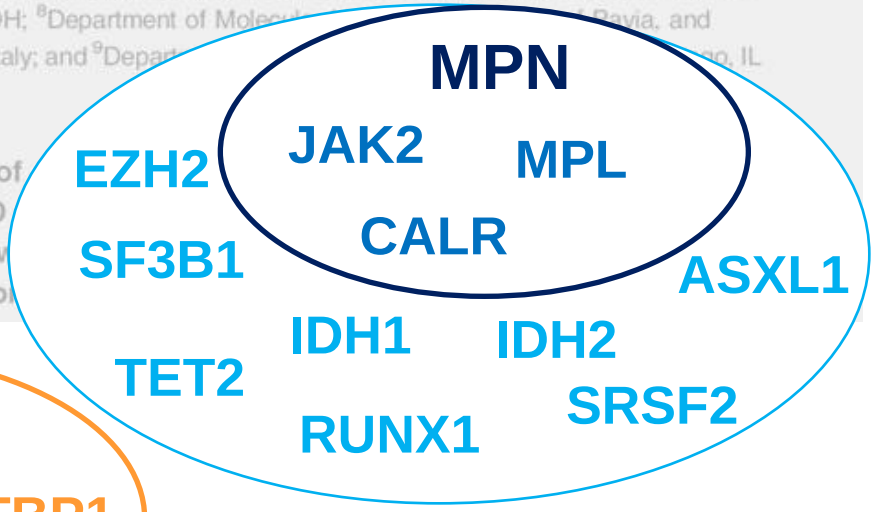
Kunnen voorkomen in meerdere/alle monsters in een run

Review Series

THE UPDATED WHO CLASSIFICATION OF HEMATOLOGICAL MALIGNANCIES

The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia





Variant calls in een patiëntenmonster

AmpliSeq AML panel op Ion Torrent PGM

Gene	Location	Pos.	Type	Coverage	c. HGVS	p. HGVS
ASXL1	E13	215 (1934) / 1bp [chr20:g.31022449 (hg19_ens)]	D	6% (292) [6% (287) / 50% (5)]	c.1934delG	p.Gly645Valfs*58
ASXL1	E13	725 (2444) [chr20:g.31022959 (hg19_ens)]	C	100% (1515) [100% (646) / 100% (869)]	c.2444T>C	p.Leu815Pro
ASXL1	E13	1587 (3306) [chr20:g.31023821 (hg19_ens)]	C	49% (704) [49% (377) / 50% (327)]	c.3306G>T	p.Glu1102Asp
CEBPA	E1	257 (107) / 1bp [chr19:g.33793214 (hg19_ens)]	D	18% (3296) [36% (2692) / 5% (604)]	c.107delG	p.Gly36Alafs*124
CEBPA	E1	263..264 (113..114) / 2bp [chr19:g.33793207_33793208 (hg19_ens)]	D	27% (5027) [69% (5014) / 0% (13)]	c.113_114delGC	p.Gly38Alafs*69
CEBPA	E1	427 (277) / 1bp [chr19:g.33793044 (hg19_ens)]	D	14% (1499) [0% (0) / 36% (1499)]	c.277delG	p.Ala93Argfs*67
CEBPA	E1	504 (354) / 1bp [chr19:g.33792967 (hg19_ens)]	D	19% (2331) [19% (1156) / 19% (1175)]	c.354delC	p.Val119Serfs*41
CEBPA	E1	643 (493) / 1bp [chr19:g.33792828 (hg19_ens)]	D	20% (4398) [42% (4320) / 1% (78)]	c.493delC	p.Arg165Alafs*153
CEBPA	E1	684 (534) / 1bp [chr19:g.33792787_33792788 (hg19_ens)]	I	11% (1083) [27% (1078) / 0% (5)]	c.533_534insG	p.Phe179Leufs*142
CEBPA	E1	879 (729) / 1bp [chr19:g.33792592 (hg19_ens)]	D	30% (2007) [0% (1) / 58% (2006)]	c.729delC	p.Ala244Profs*74
CEBPA	E1	920 (770) / 1bp [chr19:g.33792551 (hg19_ens)]	D	14% (866) [0% (0) / 23% (866)]	c.770delG	p.Gly257Alafs*61
CEBPA	E1	924 (774) / 1bp [chr19:g.33792547 (hg19_ens)]	D	25% (1561) [64% (1560) / 0% (1)]	c.774delC	p.Ala259Argfs*59
CEBPA	E1	1010 (860) / 1bp [chr19:g.33792461_33792462 (hg19_ens)]	I (Dup)	13% (131) [1% (5) / 24% (126)]	c.859dupG	p.Val287Glyfs*34
CEBPA	E1	1117 (967) / 1bp [chr19:g.33792354 (hg19_ens)]	D	18% (349) [41% (349) / 0% (0)]	c.967delC	p.Arg323Alafs*9
DNMT3A	E9	+7 [chr2:g.25469913 (hg19_ens)]	C	50% (664) [50% (336) / 49% (328)]	c.1122+7G>A	
DNMT3A	E22	100 (2578) [chr2:g.25458595 (hg19_ens)]	C	46% (326) [44% (182) / 48% (144)]	c.2578T>C	p.Trp860Arg
GATA2	E2	60 (15) [chr3:g.128205860 (hg19_ens)]	C	51% (5199) [51% (2480) / 52% (2719)]	c.15C>G	p.=
GATA2	E3	261 (490) [chr3:g.128204951 (hg19_ens)]	C	52% (615) [51% (267) / 54% (348)]	c.490G>A	p.Ala164Thr
IDH1	E4	273 (395) [chr2:g.209113112 (hg19_ens)]	C	48% (530) [48% (329) / 47% (201)]	c.395G>A	p.Arg132His
NPM1	E11	18 (864) / 4bp [chr5:g.170837547_170837548 (hg19_ens)]	I (Dup)	48% (5496) [48% (3199) / 47% (2297)]	c.860_863dupTCTG	p.Trp288Cysfs*12
RUNX1	E4	70 (167) / 1bp [chr21:g.36259324 (hg19_ens)]	D	13% (107) [25% (106) / 0% (1)]	c.167delT	p.Leu56Cysfs*16
RUNX1	E8	112 (917) / 1bp [chr21:g.36171648 (hg19_ens)]	D	26% (441) [59% (441) / 0% (0)]	c.917delG	p.Arg306Leufs*5
TET2	E11	747 (5284) [chr4:g.106196951 (hg19_ens)]	C	100% (1402) [100% (666) / 100% (736)]	c.5284A>G	p.Ile1762Val
TP53	E4	119 (215) [chr17:g.7579472 (hg19_ens)]	C	98% (1491) [98% (862) / 99% (629)]	c.215C>G	p.Pro72Arg
TP53	E4	181 (277) / 1bp [chr17:g.7579410 (hg19_ens)]	D	13% (196) [23% (196) / 0% (0)]	c.277delC	p.Leu93Cysfs*30
TP53	E5	72 (447) / 1bp [chr17:g.7578483 (hg19_ens)]	D	13% (275) [25% (275) / 0% (0)]	c.447delC	p.Thr150Hisfs*20
TP53	E5	80 (455) / 1bp [chr17:g.7578475 (hg19_ens)]	D	27% (529) [56% (518) / 1% (11)]	c.455delC	p.Pro152Argfs*18

26 variant calls

Radboudumc

Fout-positieve variant calls - errors

- Vergelijking van alle monster binnen een run (niet-random errors)
- Lijst van bekende fout-positieven (eigen ervaring, methode-specifiek)
- Duplo samples analyseren (random errors)

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ASXL1	E13	215 (1934) / 1bp [chr20:g.31022449 (hg19_ens)]	D	6% (292) [6% (287) / 50% (5)]	c.1934delG	p.Gly645Valfs*58
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ASXL1	E13	1587 (3306) [chr20:g.31023821 (hg19_ens)]	C	49% (704) [49% (377) / 50% (327)]	c.3306G>T	p.Glu1102Asp
CEBPA	E1	257 (107) / 1bp [chr19:g.33793214 (hg19_ens)]	D	18% (3296) [36% (2692) / 5% (604)]	c.107delG	p.Gly36Alafs*124
CEBPA	E1	263..264 (113..114) / 2bp [chr19:g.33793207_33793208 (hg19_ens)]	D	27% (5027) [69% (5014) / 0% (13)]	c.113_114delGC	p.Gly38Alafs*69
CEBPA	E1	427 (277) / 1bp [chr19:g.33793044 (hg19_ens)]	D	14% (1499) [0% (0) / 36% (1499)]	c.277delG	p.Ala93Argfs*67
CEBPA	E1	504 (354) / 1bp [chr19:g.33792967 (hg19_ens)]	D	19% (2331) [19% (1156) / 19% (1175)]	c.354delC	p.Val119Serfs*41
CEBPA	E1	643 (493) / 1bp [chr19:g.33792828 (hg19_ens)]	D	20% (4398) [42% (4320) / 1% (78)]	c.493delC	p.Arg165Alafs*153
CEBPA	E1	684 (534) / 1bp [chr19:g.33792787_33792788 (hg19_ens)]	I	11% (1083) [27% (1078) / 0% (5)]	c.533_534insG	p.Phe179Leufs*142
CEBPA	E1	879 (729) / 1bp [chr19:g.33792592 (hg19_ens)]	D	30% (2007) [0% (1) / 58% (2006)]	c.729delC	p.Ala244Profs*74
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CEBPA	E1	1117 (967) / 1bp [chr19:g.33792354 (hg19_ens)]	D	18% (349) [41% (349) / 0% (0)]	c.967delC	p.Arg323Alafs*9
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DNMT3A	E22	100 (2578) [chr2:g.25458595 (hg19_ens)]	C	46% (326) [44% (182) / 48% (144)]	c.2578T>C	p.Trp860Arg
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IDH1	E4	273 (395) [chr2:g.209113112 (hg19_ens)]	C	48% (530) [48% (329) / 47% (201)]	c.395G>A	p.Arg132His
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TP53	E5	80 (455) / 1bp [chr17:g.7578475 (hg19_ens)]	D	27% (529) [56% (518) / 1% (11)]	c.455delC	p.Pro152Argfs*18

Variant Interpretatie – polymorfismen

- Polymorfismen: VAF 50% (heterozygoot) of 100% (homozygoot)
- dbSNP en populatie databases
- Lijst van frequent voorkomende polymorfismen

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TP53	E5	80 (455) / 1bp [chr17:g.7578475 (hg19_ens)]	D	27% (529) [56% (518) / 1% (11)]	c.455delC	p.Pro152Argfs*18

Variant Interpretatie – mutaties

- Duplo samples analyseren
- Hotspot mutaties: bekende pathogene mutaties (literatuur, databases)
- Database search

Gene	Location	Pos.	Type	Coverage	c. HGVS	p. HGVS
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ASXL1	E13	725 (2444) [chr20:g.31022959 (hg19_ens)]	C	100% (1515) [100% (646) / 100% (869)]	c.2444T>C	p.Leu815Pro
ASXL1	E13	1587 (3306) [chr20:g.31023821 (hg19_ens)]	C	49% (704) [49% (377) / 50% (327)]	c.3306G>T	p.Glu1102Asp
CEBPA	E1	257 (107) / 1bp [chr19:g.33793214 (hg19_ens)]	D	18% (3296) [36% (2692) / 5% (604)]	c.107delG	p.Gly36Alafs*124
CEBPA	E1	263..264 (113..114) / 2bp [chr19:g.33793207_33793208 (hg19_ens)]	D	27% (5027) [69% (5014) / 0% (13)]	c.113_114delGC	p.Gly38Alafs*69
CEBPA	E1	427 (277) / 1bp [chr19:g.33793044 (hg19_ens)]	D	14% (1499) [0% (0) / 36% (1499)]	c.277delG	p.Ala93Argfs*67
CEBPA	E1	504 (354) / 1bp [chr19:g.33792967 (hg19_ens)]	D	19% (2331) [19% (1156) / 19% (1175)]	c.354delC	p.Val119Serfs*41
CEBPA	E1	643 (493) / 1bp [chr19:g.33792828 (hg19_ens)]	D	20% (4398) [42% (4320) / 1% (78)]	c.493delC	p.Arg165Alafs*153
CEBPA	E1	684 (534) / 1bp [chr19:g.33792787_33792788 (hg19_ens)]	I	11% (1083) [27% (1078) / 0% (5)]	c.533_534insG	p.Phe179Leufs*142
CEBPA	E1	879 (729) / 1bp [chr19:g.33792592 (hg19_ens)]	D	30% (2007) [0% (1) / 58% (2006)]	c.729delC	p.Ala244Profs*74
CEBPA	E1	920 (770) / 1bp [chr19:g.33792551 (hg19_ens)]	D	14% (866) [0% (0) / 23% (866)]	c.770delG	p.Gly257Alafs*61
CEBPA	E1	924 (774) / 1bp [chr19:g.33792547 (hg19_ens)]	D	25% (1561) [64% (1560) / 0% (1)]	c.774delC	p.Ala259Argfs*59
CEBPA	E1	1117 (967) / 1bp [chr19:g.33792354 (hg19_ens)]	D	18% (349) [41% (349) / 0% (0)]	c.967delC	p.Arg323Alafs*9
DNMT3A	E9	+7 [chr2:g.25469913 (hg19_ens)]	C	50% (664) [50% (336) / 49% (328)]	c.1122+7G>A	
DNMT3A	E22	100 (2578) [chr2:g.25458595 (hg19_ens)]	C	46% (326) [44% (182) / 48% (144)]	c.2578T>C	p.Trp860Arg
GATA2	E2	60 (15) [chr3:g.128205860 (hg19_ens)]	C	51% (5199) [51% (2480) / 52% (2719)]	c.15C>G	p.=
GATA2	E3	261 (490) [chr3:g.128204951 (hg19_ens)]	C	52% (615) [51% (267) / 54% (348)]	c.490G>A	p.Ala164Thr
IDH1	E4	273 (395) [chr2:g.209113112 (hg19_ens)]	C	48% (530) [48% (329) / 47% (201)]	c.395G>A	p.Arg132His
NPM1	E11	18 (864) / 4bp [chr5:g.170837547_170837548 (hg19_ens)]	I (Dup)	48% (5496) [48% (3199) / 47% (2297)]	c.860_863dupTCTG	p.Trp288Cysfs*12
RUNX1	E4	70 (167) / 1bp [chr21:g.36259324 (hg19_ens)]	D	13% (107) [25% (106) / 0% (1)]	c.167delT	p.Leu56Cysfs*16
RUNX1	E8	112 (917) / 1bp [chr21:g.36171648 (hg19_ens)]	D	26% (441) [59% (441) / 0% (0)]	c.917delG	p.Arg306Leufs*5
TET2	E11	747 (5284) [chr4:g.106196951 (hg19_ens)]	C	100% (1402) [100% (666) / 100% (736)]	c.5284A>G	p.Ile1762Val
TP53	E4	119 (215) [chr17:g.7579472 (hg19_ens)]	C	98% (1491) [98% (862) / 99% (629)]	c.215C>G	p.Pro72Arg
TP53	E4	181 (277) / 1bp [chr17:g.7579410 (hg19_ens)]	D	13% (196) [23% (196) / 0% (0)]	c.277delC	p.Leu93Cysfs*30
TP53	E5	72 (447) / 1bp [chr17:g.7578483 (hg19_ens)]	D	13% (275) [25% (275) / 0% (0)]	c.447delC	p.Thr150Hisfs*20
TP53	E5	80 (455) / 1bp [chr17:g.7578475 (hg19_ens)]	D	27% (529) [56% (518) / 1% (11)]	c.455delC	p.Pro152Argfs*18

Variant Interpretatie – onbekende varianten (1)

Variant DNMT3A c.2578T>C p.Trp860Arg VAF 46%

Niet een bekend polymorfisme

Niet de bekende DNMT3A hotspot mutatie (p. Arg882His)

Database search m.b.v. **Alamut**

software applicatie die informatie van verschillende databases en andere bronnen combineert ten behoeve van variant interpretatie

- dbSNP (polymorfismen)
- populatie databases; o.a. EXAC, ESP, GoNL
- COSMIC (somatische mutaties)
- ziekte-variant correlaties (ClinVar, HGMD, SwissProt)
- Voorspellingssoftware mutatie effect (SIFT, MutationTaster, PolyPhen)
- Evolutionair geconserveerd nucleotide, aminozuur
- Eiwitdomeinen

Variant Interpretatie – onbekende varianten (2)

Report for mutation **NM_175629.2(DNMT3A):c.2578T>C**

Warning: *This report is based on knowledge and data that are not firmly established. Consequently, medical decisions must not be made on the basis of this report.*

DNMT3A Variation

alamut visual

Transition from T to C in exon 22.
Missense substitution.
Trp at position 860 is changed to Arg.

This variant is known to dbSNP (147): rs373014701 (not validated dbSNP entry).

This variant is known to ESP (ESP6500SIV2): Eur. Am.: G=0.01% - Afr. Am.: G=0.00%

This variant is known to ExAC (1.0): ALL:C=0.0066% - AFR:0% - AMR:0% - EAS:0% - SAS:0% - NFE:0.010% - FIN:0.015% - OTH:0% (**Filter:** VQSRTrancheINDEL99.00to99.50)

HGVS v2.0 Nomenclature

cDNA Level:	**NM_175629.2:c.2578T>C**
gDNA Level:	**Chr2(GRCh37):g.25458595A>G**
Protein Level:	**p.Trp860Arg**

Pathogenicity clues

- Highly conserved nucleotide (phyloP: 4.89 [-14.1;6.4])
- Highly conserved amino acid, up to Zebrafish (considering 12 species)
- Moderate physicochemical difference between Trp and Arg (Grantham dist.: 101 [0-215])
- Align GVGD: C0 (GV: 268.54 - GD: 82.54)
- SIFT: Deleterious (score: 0, median: 4.32)
- MutationTaster: disease causing (p-value: 1)

Variant Interpretatie – onbekende varianten (3)

Is de DNMT3A variant een (zeldzaam) polymorfisme (SNP)?

VAF 46% consistent met heterozygote erfelijke/kiembaan variant

Komt voor in populatie databases:

dbSNP (not validated SNP entry)

EXAC MAF 0,0066%

ESP MAF 0,01%

Testen constitutioneel DNA (speeksel, wangslijmvlies)

Speeksel: DNMT3A c.2578T>C VAF 2%

→ DNMT3A variant is een **verworven mutatie**

Variant Interpretatie – onbekende varianten (4)

Home ▾ | Resources ▾ | Curation ▾ | Tools ▾ | Data ▾ | News ▾ | Help ▾ | About ▾

Cosmic » Mutation » Overview » DNMT3A p.W860R / c.2578T>C

Overview | Tissue Distribution | Samples | Pathways Affected | References

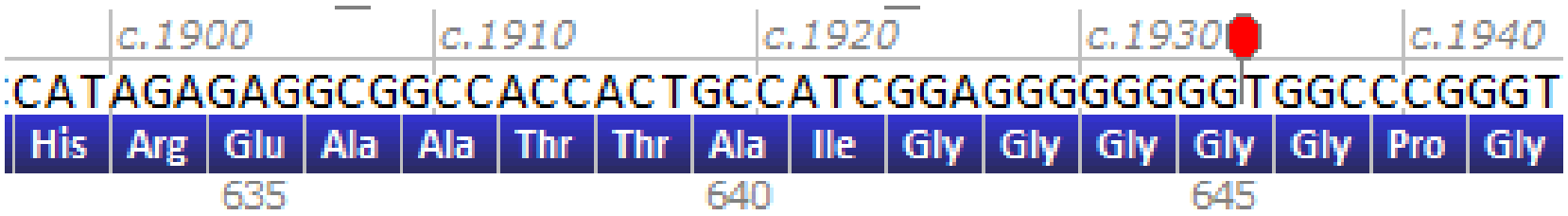
Gene Name:	DNMT3A
Mutation Id:	COSM231568
AA Mutation:	p.W860R (Substitution - Missense, position 860, W→R)
CDS Mutation:	c.2578T>C (Substitution, position 2578, T→C)
GRCh38:	2:25235726..25235726, view Ensembl Contig
COSMIC Genome Browser:	2:25235726..25235726, view in COSMIC JBrowse
CDD:	NP_783328.1
Ever confirmed somatic:	Yes
FATHMM prediction:	Pathogenic (score 0.99)

Variant is gerapporteerd in 6 samples (somatisch)

Variant bescheven als verworven mutatie in AML, MDS

Gen-specifieke issues

- ASXL1 hotspot c.1934dupG



- Grotere inserties/deleties: kunnen gemist worden doordat primer op positie van de ins/del ligt

Bv. CALR 52 bp del (type 1), ASXL1 c.1900_1922del23

Bij grote inserties/deleties is alignment moeilijk

Bv. FLT3-ITD

- Specieke genen kunnen moeilijk te sequencen zijn met NGS

Bv. CEBPA

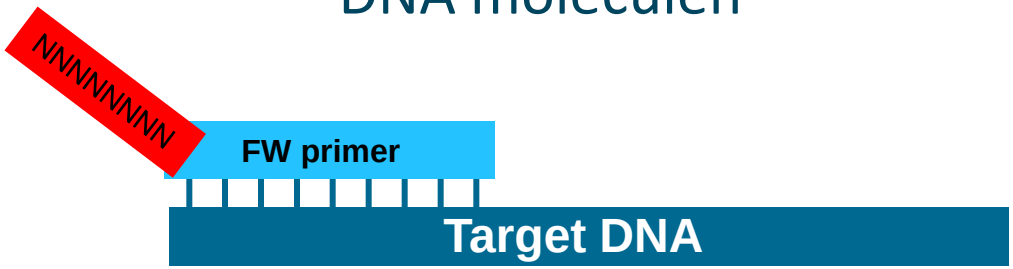
Uitdaging voor NGS in kanker

Betrouwbare en gevoelige detectie van varianten op laag niveau

ZONDER overmaat aan fout-positieve calls

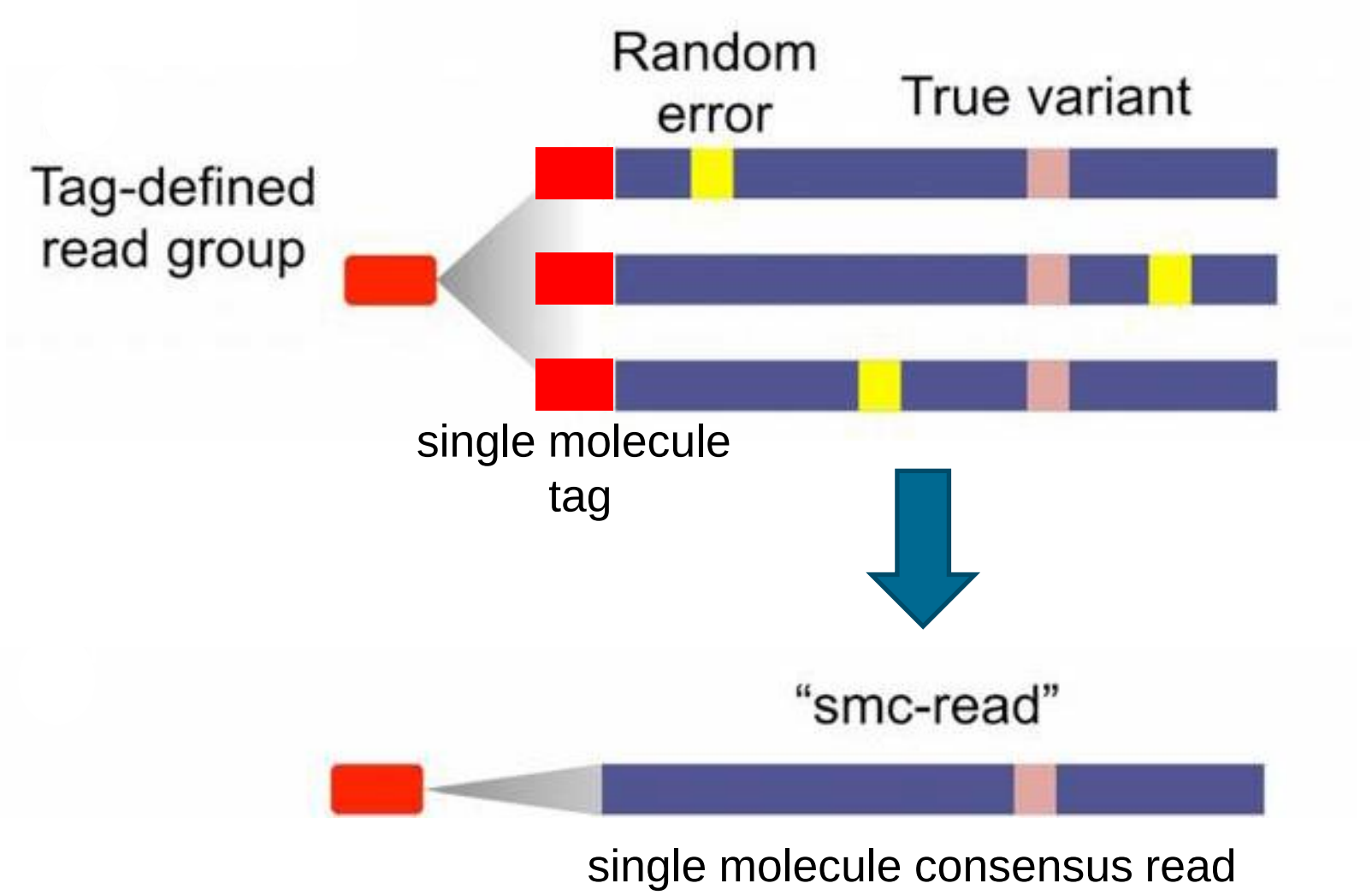
Error-corrected sequencing

Primers (PCR) or probes (capture) zijn uitgerust met een **random nucleotide tag** voor de identificatie van unieke captures van target DNA moleculen



- Alle reads van één random nucleotide tag worden gecombineerd tot één consensus sequentie om random errors uit te sluiten
- Analyse van unieke captures elimineert amplificatie bias

Random nucleotide tags - Error-correctie



Moleculaire Diagnostiek mbv NGS

- **Sequencing platform**
- **Gen panel(s);** commercieel, custom design, in-house design
- **Analyse software**; basecalling, read mapping/alignment, variant calling en annotatie
- **Coverage analysis, 'dekkingsgraad'** → mutatie detectie grens, gevoeligheid
- **Filteren van varianten**; false positive calls vs true positive calls
- **Interpretatie van varianten**; polymorfismen, onbekende varianten, relevante mutaties

Conclusie

- Goede en uitvoerige validatie van sequencing platform, analyse software en gebruikte genpanel is essentieel
- Filteren en interpreteren van gevonden varianten
  → klinisch relevante mutaties
- www.modhem.nl: aanbevelingen NGS, en aanbevelingen mutaties bij myeloïde maligniteiten
- Error-corrected sequencing kan de gevoeligheid van mutatiedetectie verhogen en errors en amplificatie bias verminderen
- Juni 2017: MODHEM rondzending NGS (TP53)