Genetic methods

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Molecular genetic diagnosis of diseases

Type of Test	Indications	Analytical Validity	Clinical Validity	Clinical Utility	Ethical, Legal, Social Issues
Diagnosis of Genetic Disorders					
Cytogenomics	Diagnosis of individual with multiple congenital anomalies, intellectual disability, signs of chromosomal abnormality	Current standards are to only report copy number changes >400 kb pairs in size	Requires validation of pathogenicity, as some CNVs are benign variants	Diagnosis, counseling	May detect CNV in parents of affected child
Disease-targeted testing	Suspected genetic disorder based on signs and symptoms, risk of inheriting genetic disorder based on family history, risk of being carrier for autosomal-recessive trait based on ancestry	Clinical laboratories must assess analytical validity for assays used for clinical reporting	May be unable to detect all possible pathogenic mutations, variants of unknown significance may be difficult to interpret	Diagnosis, counseling; may provide guidance toward surveillance for complications and/or specific therapy	Potential for stigmatization, anxiety, guilt for those found to carry mutation
Exome/genome sequencing	Suspected genetic disorder based on signs and symptoms but no known single gene condition fits phenotype	Mutations suspected as being pathogenic should be validated by Sanger sequencing	May reveal a previously annotated pathogenic mutation or a mutation that can be shown to be pathogenic; requires bioinformatic analysis to filter large number of variants, with high chance of finding variants of unknown significance	Diagnosis, counseling, end to "diagnostic odyssey"; in some cases may provide guidance toward surveillance for complications and/or specific therapy	Potential identification of incidental findings that are medically significant

JAMA, April 10, 2013—Vol 309, No. 14

Genetikai vizsgálatok etikai/jogi szabályozása

2008. évi XXI. törvény

a humángenetikai adatok védelméről, a humángenetikai vizsgálatok és kutatások, valamint a biobankok működésének szabályairól

Magyar Közlöny, 2009, 193, 47449

A laboratórium által végzett, és hazai, vagy nemzetközi Külső Minőségellenőrzési Rendszerben elérhető valamennyi beavatkozás esetében, minimum évente 4x, ahol ez a minőségügyi szolgáltaltó szolgáltatási sajátosságai miatt nem elérhető, minimum évente 2x részvétel és megfelelés.

Minőségirányítási kézikönyv

NAT akkreditácoó (legkésőbb 2013. január 1-jétől

USA: The Genetic Information Nondiscrimination Act (GINA) May 2008.

GINA makes it **illegal** for **health insurers to deny insurance coverage** or charge a higher rate or premium to an **otherwise healthy individual found to have a potential genetic condition or genetic predisposition towards a disease or disorder**.

GINA also makes it **illegal** for employers to use an **employee's genetic information when making hiring, firing, placement, or promotion decisions**.

Approach for genetic testing

- ***Monogenic disorders***:
  - known inheritance
  - one gene
  - mutations: rare genetic variants
  - detection: DNA sequence analysis including screening methods (SSCP, TTGE, DGGE, DNA sequencing) –today is replaced with DNA seq, NGS

- ***Complex diseases:***
  - no specific inheritance
  - Multiple genes, or polymorphisms
  - polymorphisms (SNP): frequent alterations
  - Methods: high throughput technologies: (SNP microarray, exome, whole genome DNA sequencing)

Possibilities for genetic testing of monogenic disorders

To ensure the diagnosis:

precise clinical diagnosis, including pedigree analysis in order to demonstrate the inheritance (clinical geneticist).

molecular biological methods for detection of pathogenic alterations *(molecular biological laboratory)*.

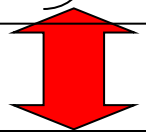
for genetic positive cases genetic counseling and screening of family members

Treatment based on genetic test: *clinical and laboratory screening for early detection, and Preventive therapeutical interventions*

Long term follow-up in mutation positive families

Family programming: genetic counseling

Team work: medical doctors from various specialties, and genetic counselors and molecular biologists….



***Complex* diseases**

Risk alleles, genetic counseling

Genetic alterations

Mutation: alteration in DNA sequence which can alter the coding sequence resulting in a defected protein

***Epidemiological definition**:*

mutation, genetic alteration whom prevalence < 1 %, 0.1%
polymorphism (SNP): prevalence > 6 %,
genetic variant: prevalence 1-6 %

Classification of mutations:

By size: point mutation (one basepair altered)
chromosome mutation (large genomic sequence, whole chromosome arm involved)

By structure:
substitution mutation,
deletions, insertions
rearrangements (intragenetically, intrachromosomal, inter-chromosomal)

By origin:
spontaneous or induced (introducing mutators: chemical substances, radiations

Characteristics of point mutations

Most of the disease causing mutations are point mutations

Types:

- *samesense mutation (synonymous, silent mutations)*: usually don't result in any changes and functionally don't have any effect. Most of the gene polymorphisms belong to this group.

-*nonsense mutations*: result is a stop codon, therefore in a shortened protein. These are loss-of function mutations.

- *missense mutations*: result in aminoacid change and altered protein function. TGC634CGC (Cys634Arg) of ***RET*** **protooncogene resulting in MEN2 syndrome)**

Detection of point mutations

***Polymerase chain reaction (PCR*)**: amplification of gene region with interest

Ingredients/Perform: DNA (isolated from peripheral blood)
Primer pairs: oligonucleotides specific for DNA region (melting temperature)
Polymerase enzyme (Taq, proof reading enzyme Pfu …)
dNTP mix
buffer, DMSO

***Screening methods*:**

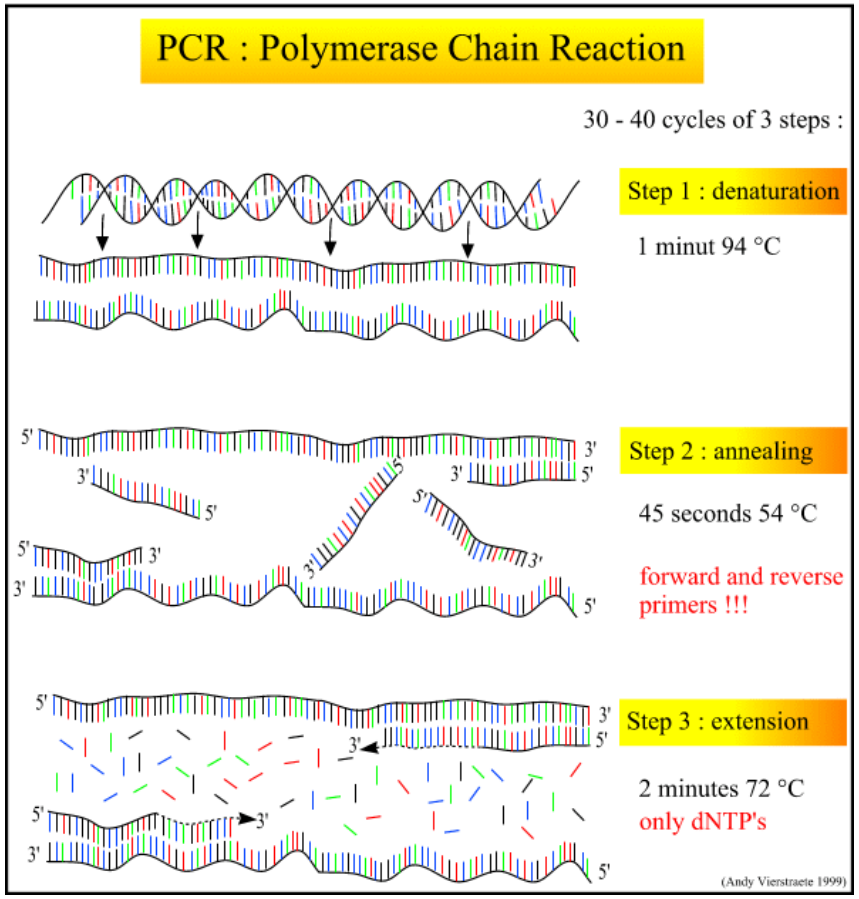
Properties: large volume, relative cheap

Methods: allele specific amplification
restriction enzyme digestion
Single strand conformation polymorphism (SSCP)
denature gradient gel electrophoresis (DGGE)
Temperature gradient gel electrophoresis (TTGE)

DNA sequencing

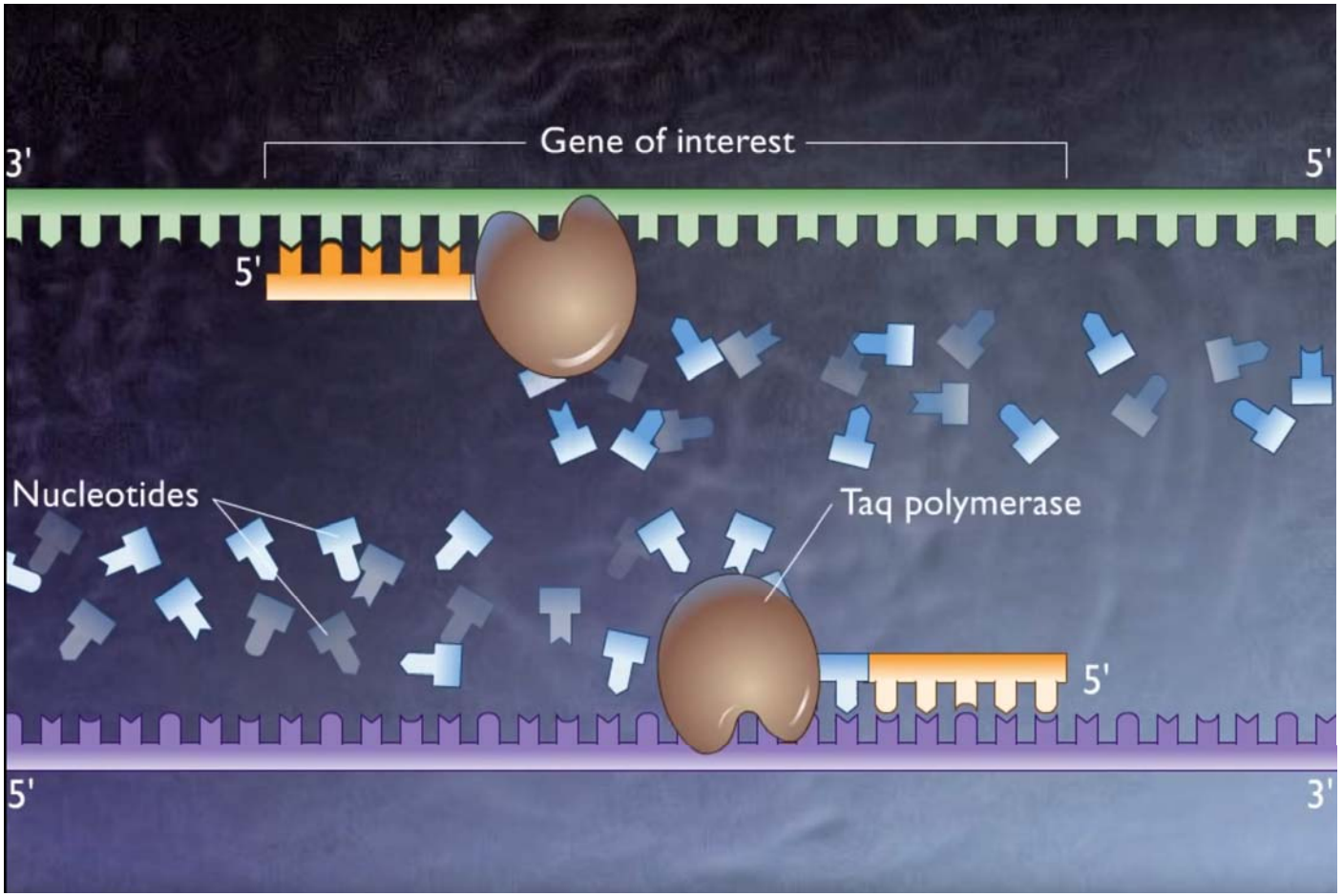
Polymerase chain reaction (PCR)

Key ingredient: Taq polymerase isolated from heat resistant bacteria



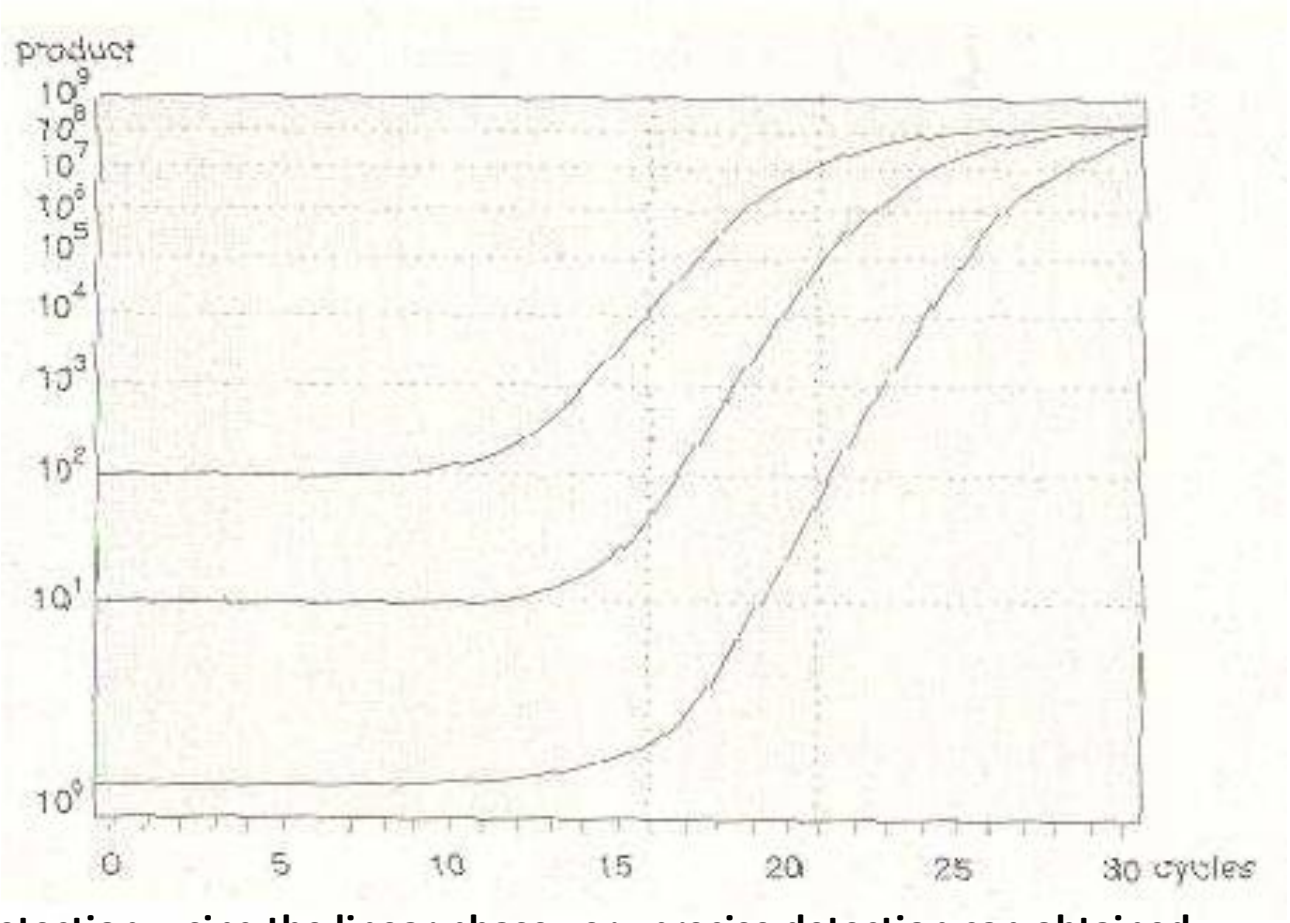
Yellowstone Nat Park, Wyoming, USA, Old Faithful geyser

Polymerase Chain Reaction



https://www.youtube.com/watch?v=MyLrs_h1OlE

Association between the PCR cycles and quantity of the PCR product



Real-time detection: using the linear phase very precise detection can obtained

Use: identification of gene deletions, evaluation of gene expression levels

eg. heterozygote deletion (hemizygote states): CYP21A2, vhl, SDHB, SDHD, menin

Screening methods, which method is the best for my goal?

Possibilities

Looking for **unknown genetic alterations** or detection of a **known alteration**

single strand conformation analysis (SSCP)

denaturant gradient gel electrophoresis (DDGE)

temporal temperature gradient electrophoresis (TTGE)

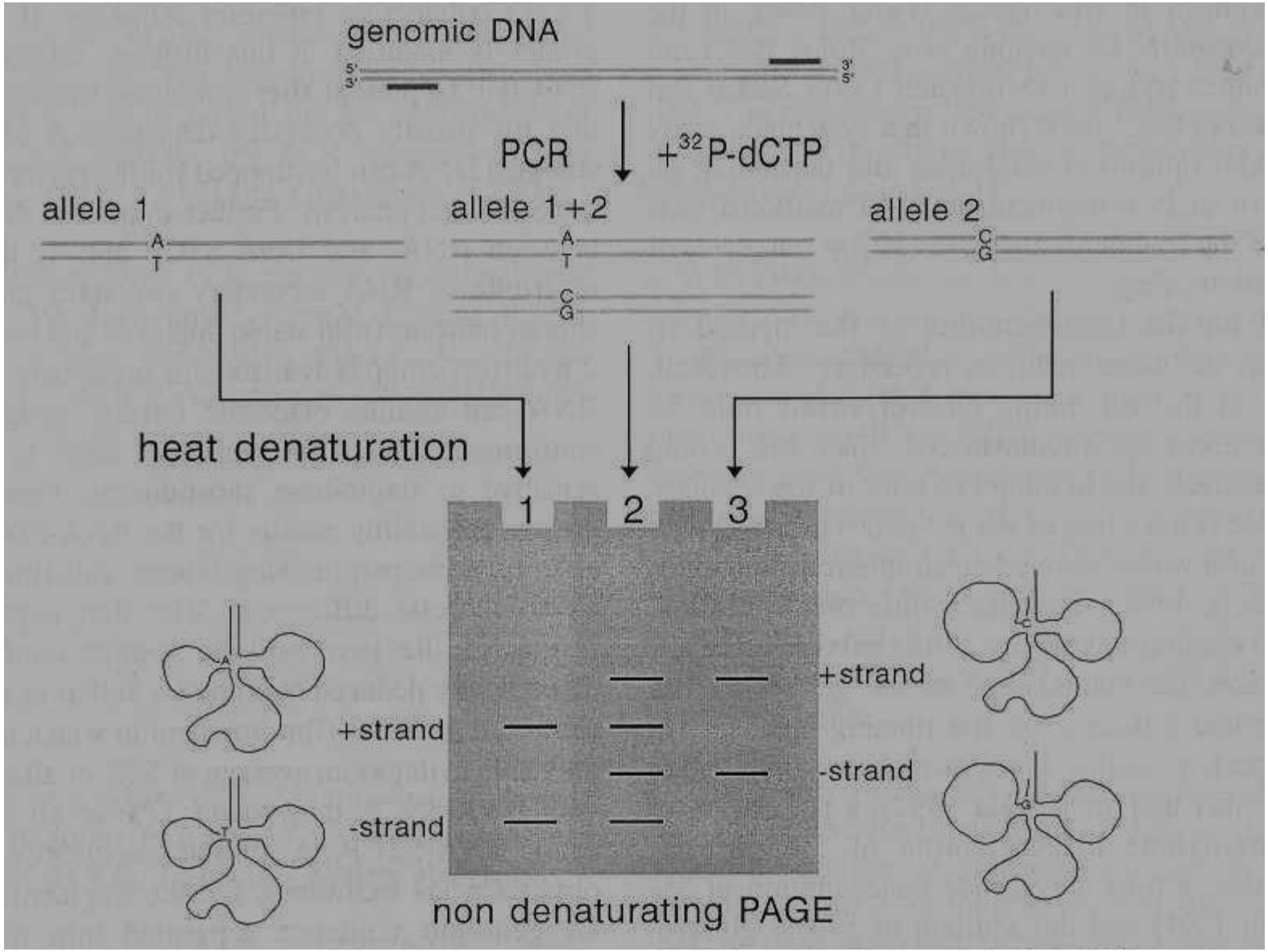
Denature high pressure liquid chromatography (DHPLC)

Allele specific amplification

Allele specific hidridization

Restriction fragment lenght polymorphism (RFLP)

Single strand conformation polymorphism (SSCP) analysis



Denature, highpressure liquid chromatography, (DHPLC)

Use: Ideal for genetic screening of mutations of large genes

Principle:	Different melting profiles of homo and heteroduplexes
Benefits:	Very good sensitivity
	Very fast (aprox. 192 samples /h)
	After preparation of PCR no need for other specific material
	Ideal for analysis of small fragments (less <200 bp)
Disadvantage:	very expansive
	It is screening method, therefore in order to identify the genetic alteration in positive cases should perform DNA sequencing

Detection of known genetic alterations

Allele specific PCR

Principle: *Specific p*rimers for amplification of a wild type and a mutant alleles in a separate PCR tubes

Every PCR reaction should contain an *internal control*:
one primer pair specific for other DNA sequence

In Human genome apr. 1 SNP/ 1000 basepair,

Use:

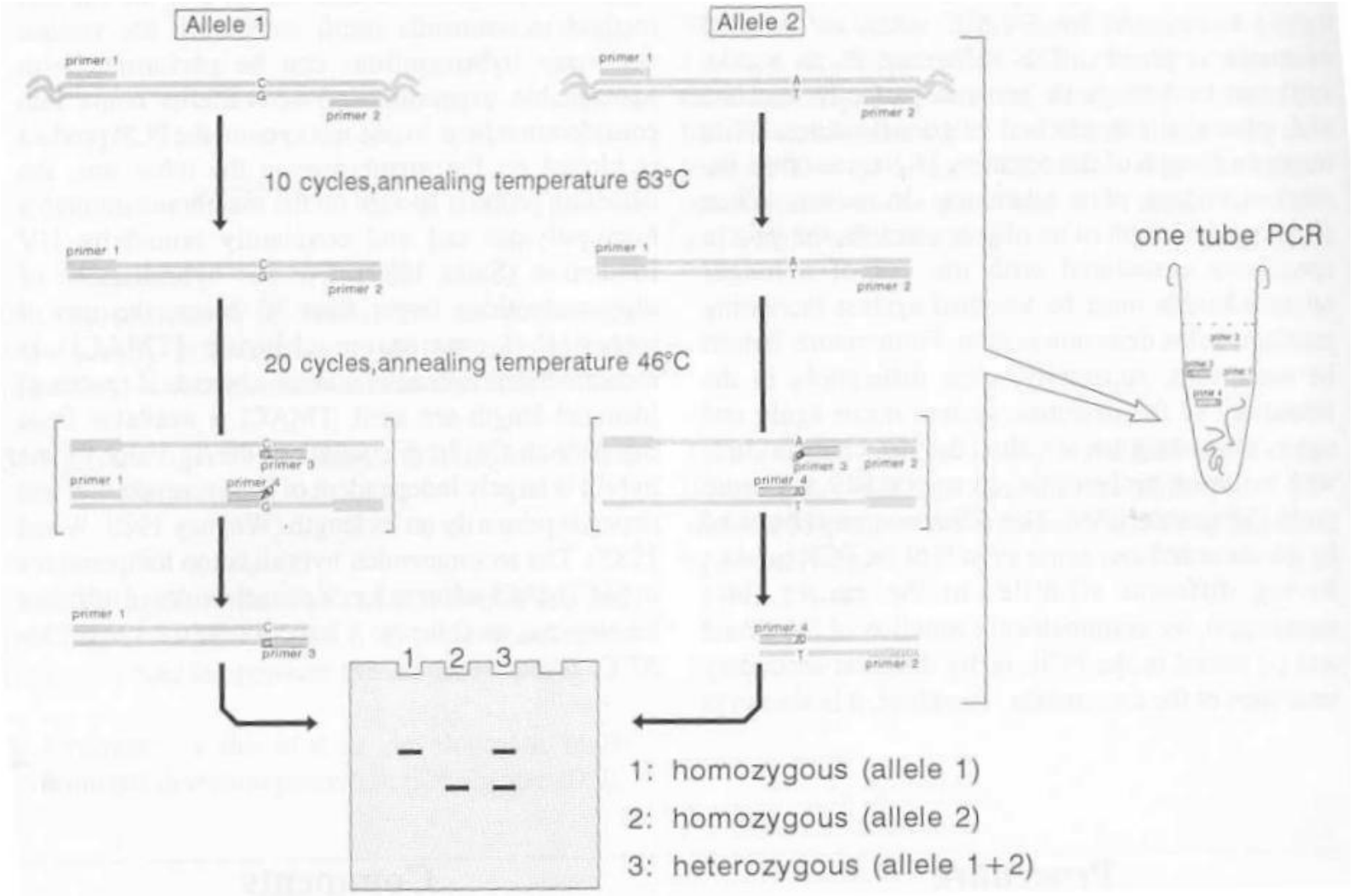
One-plex-Simple form:

- detection of known mutation of i.e. *CYP21A2* gene

Multiplex form:

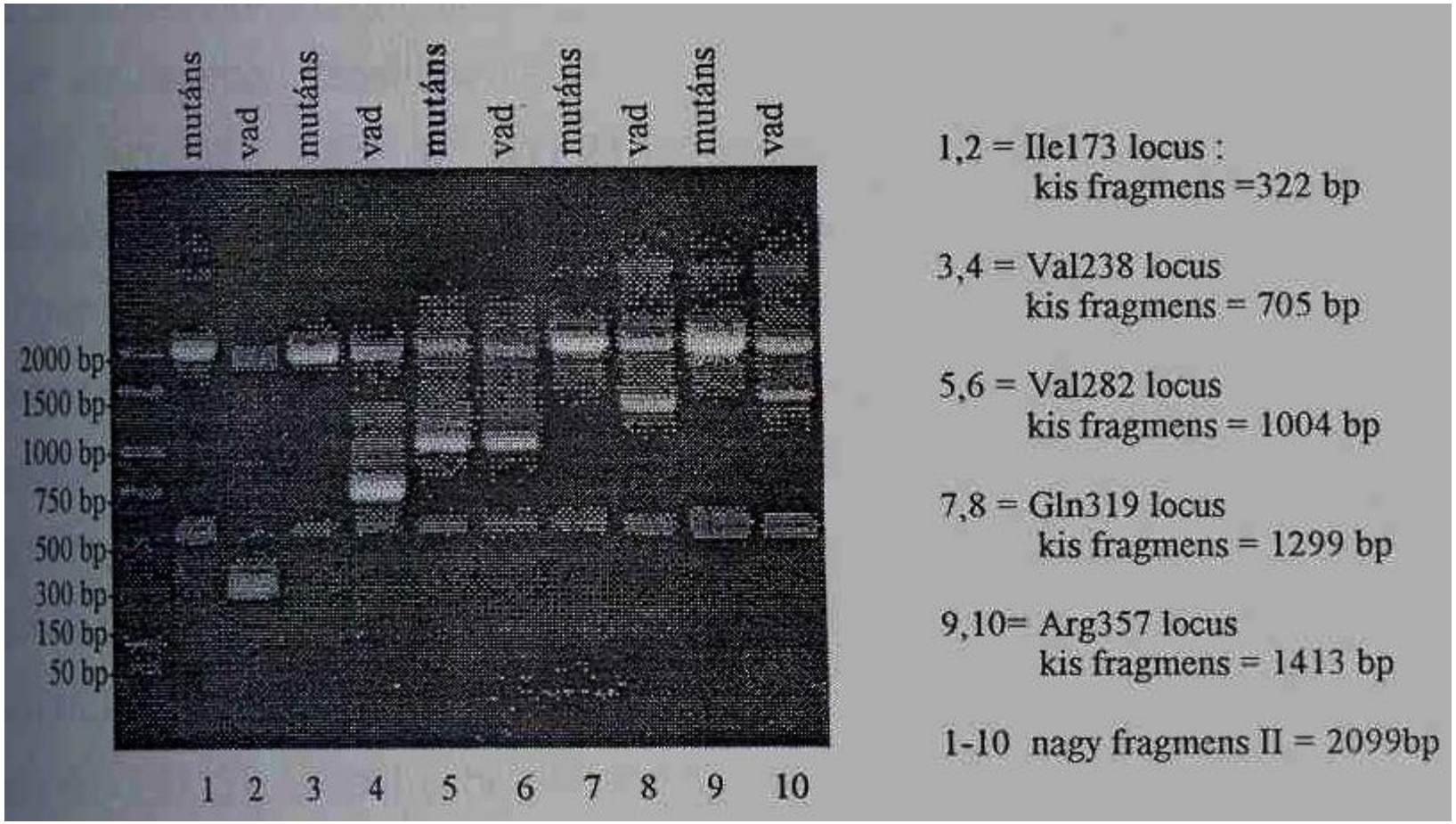
- Gene map, genetic association studies, gene hunt
high throughput technology (Illumina System, Affymetrix

Allele specific PCR



Allele specific amplification: Clinical use:

detection of mutations of the *CYP21A2* gene in patients with congenital adrenal hyperplasia



Real Time PCR for allele discrimination (RT-PCR)

Roche

Light Cycler

Kapillárisban

Roche próbák

Taqman

Sybergreen

IVD

cobas® 4800 System

96/382-es plate

Többféle jelölés

IVD

ABI

7500PCR

96-os plate

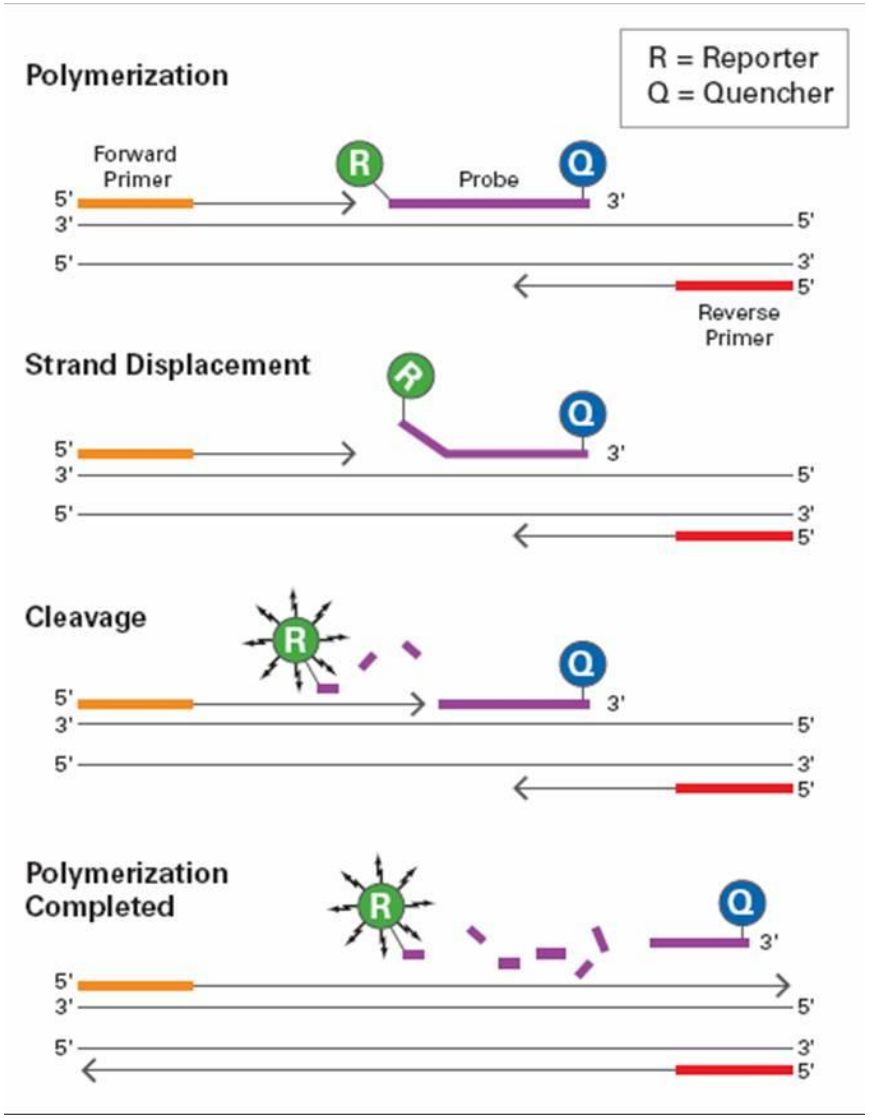
Próbák (6 féle jelölés)

Taqman

Sybergreen

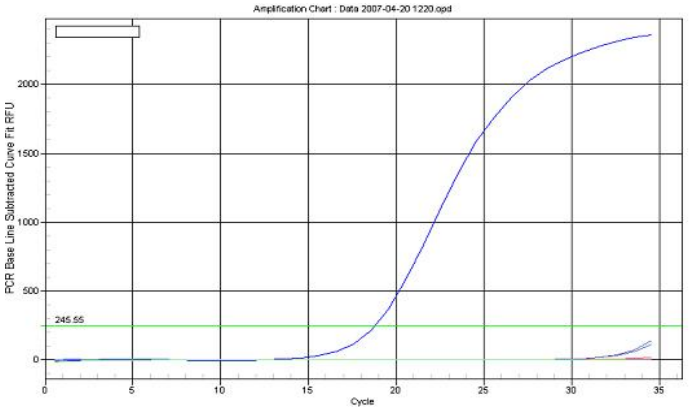
Research Use Only

Taqman chemistry



SNP two nucleotides labeled with different fluoroform (FAM, Vic)

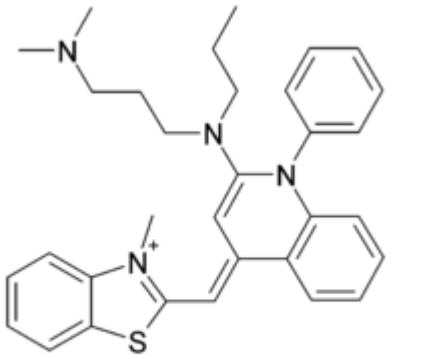
Signal will be detected only where the perfect hybridization occurs



Sybergreen labeling

DYe which intercalates between two arms of DNA → Replace Etidium bromidea ⟺ BUT

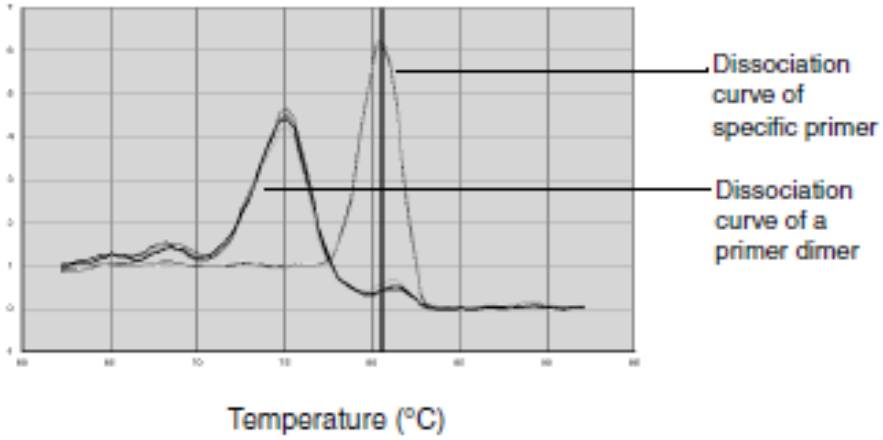
SYBR Green I



IUPAC name [hide]

N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine

Aspecific PCR, primer dimer



Confirmation of the gene alteration

Screening methods: both sensitivity and specificity < 100%

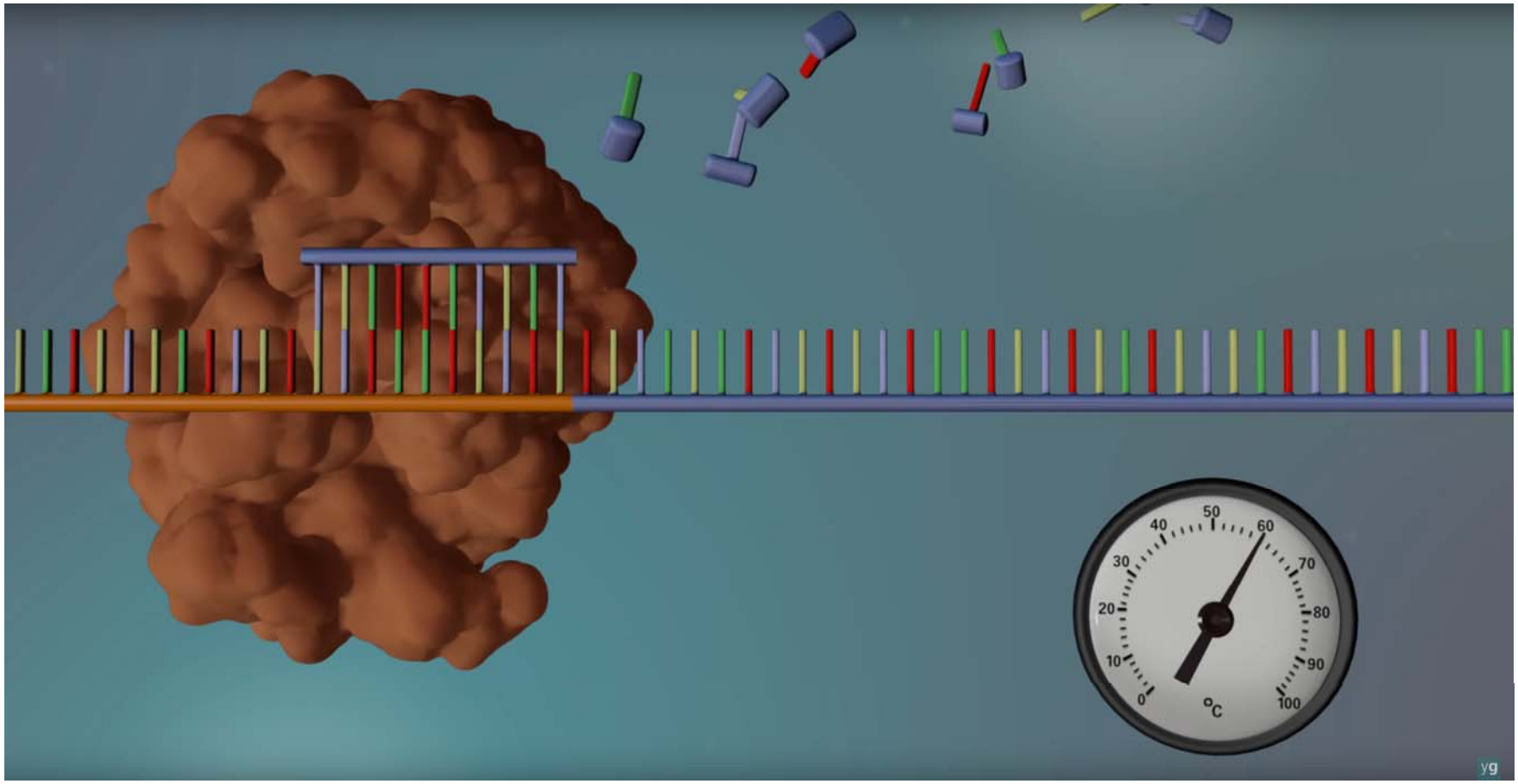
Results of the screening methods **must be confirmed**

Clinical features and the observed genetic alteration does not match, further investigations are needed

DNA sequencing:

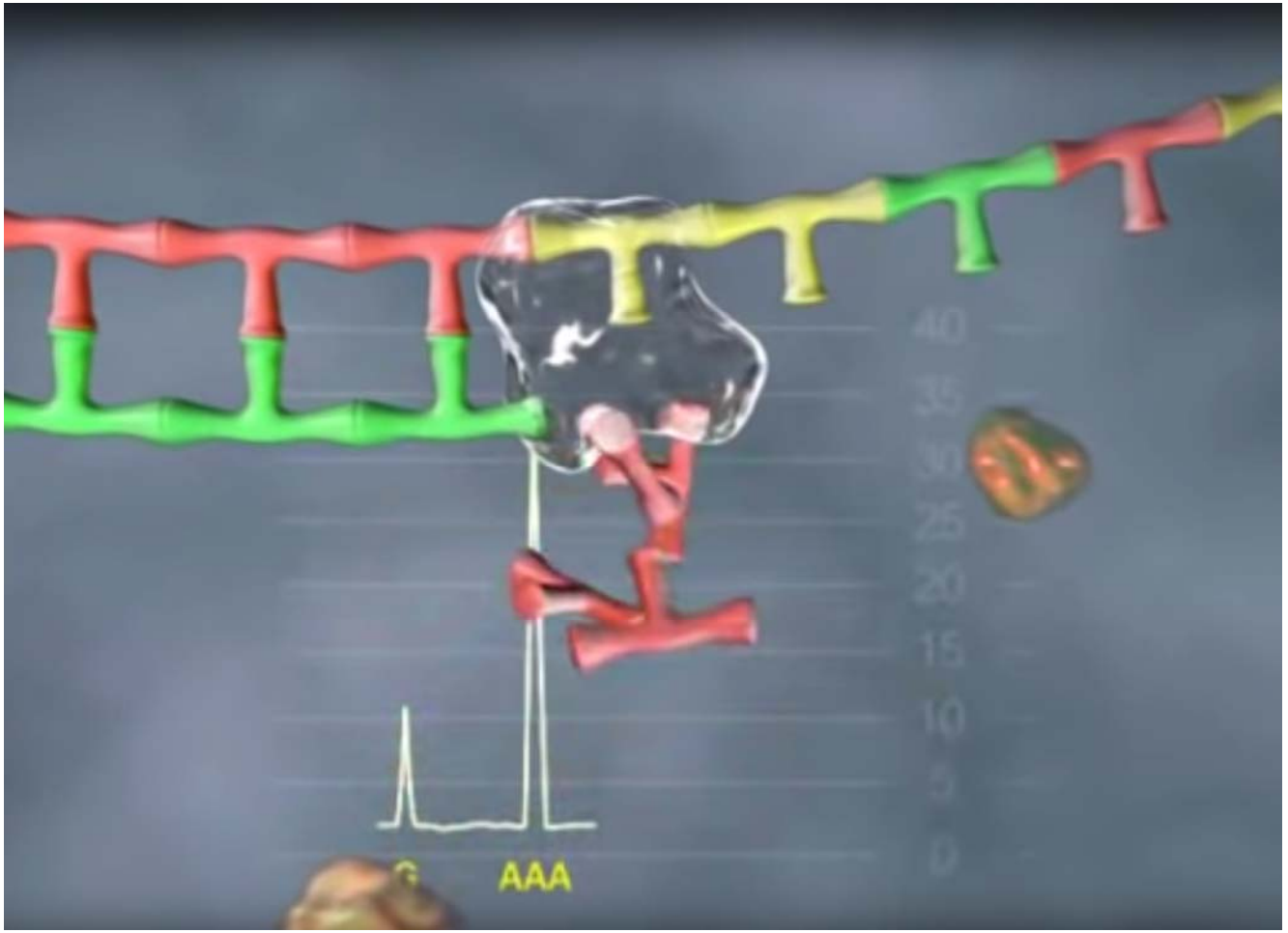
every screening method should be followed by a control method for identification and clarification of mutations

DNA Sequencing - 3D



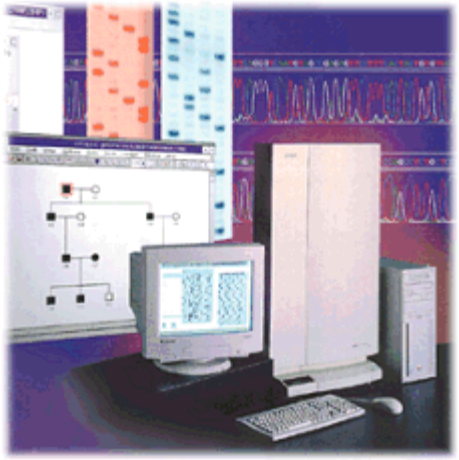
https://www.youtube.com/watch?v=ONGdehkB8jU

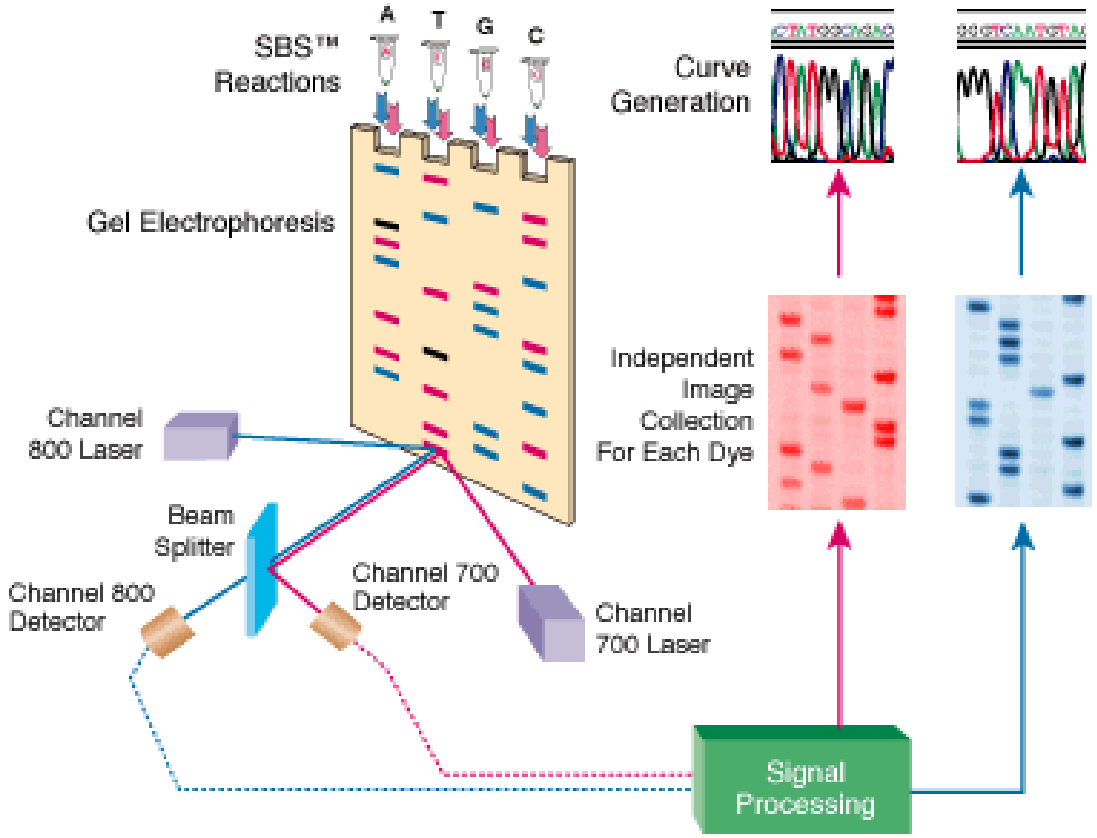
Pyro Sequencing

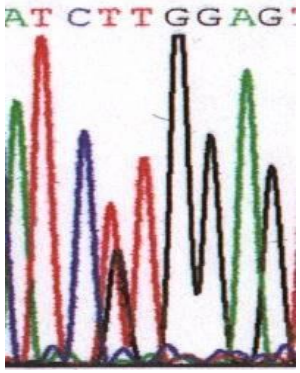


https://www.youtube.com/watch?v=nFfgWGFe0aA

DNA sequencing using dideoxy chain termination and gel or capillary electrophoresis

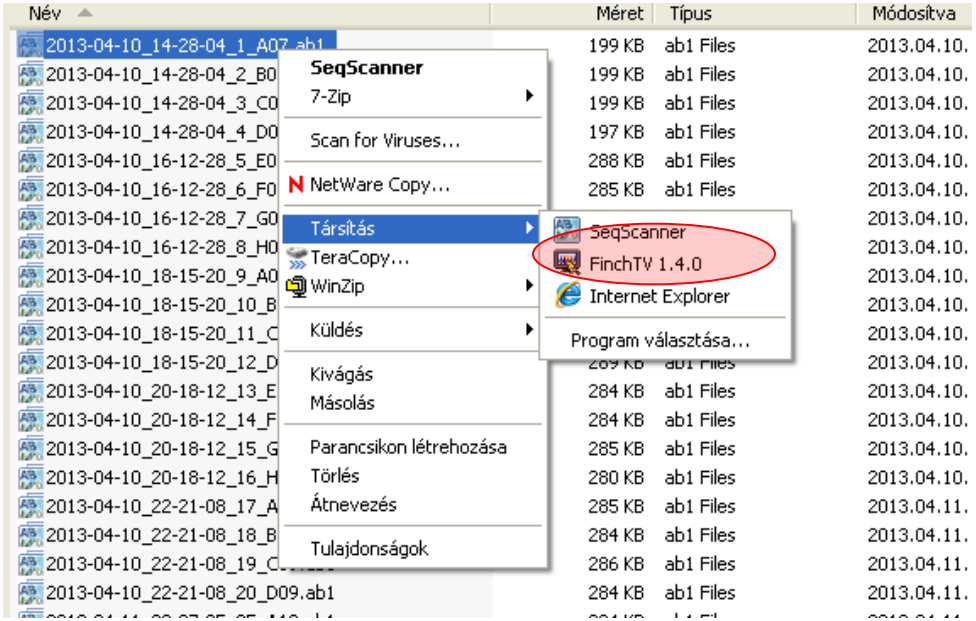


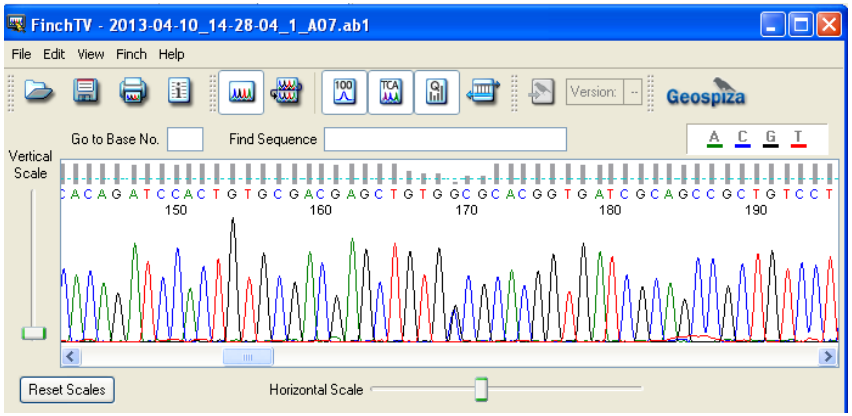




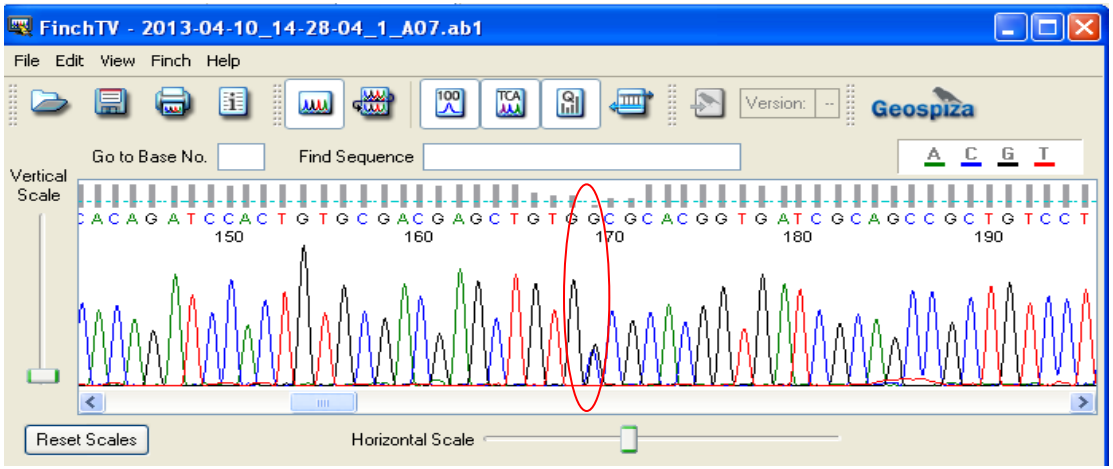
RET mutation, exon 14, CGT804CTT, Val804Leu

Analysising sequencing data

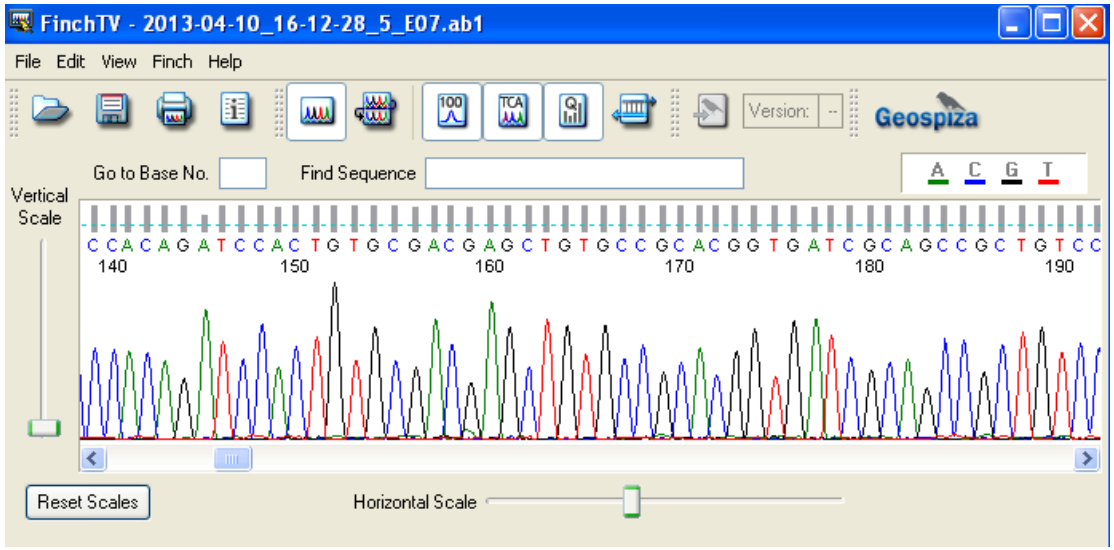




Analysing sequencing data



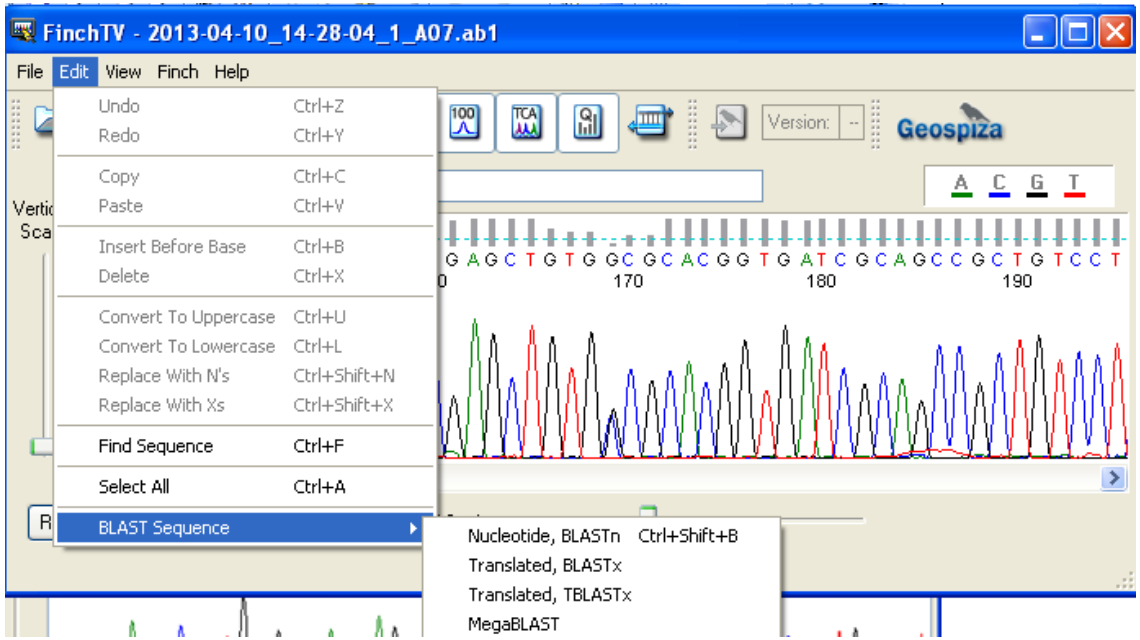
Mutation in heterozygote form

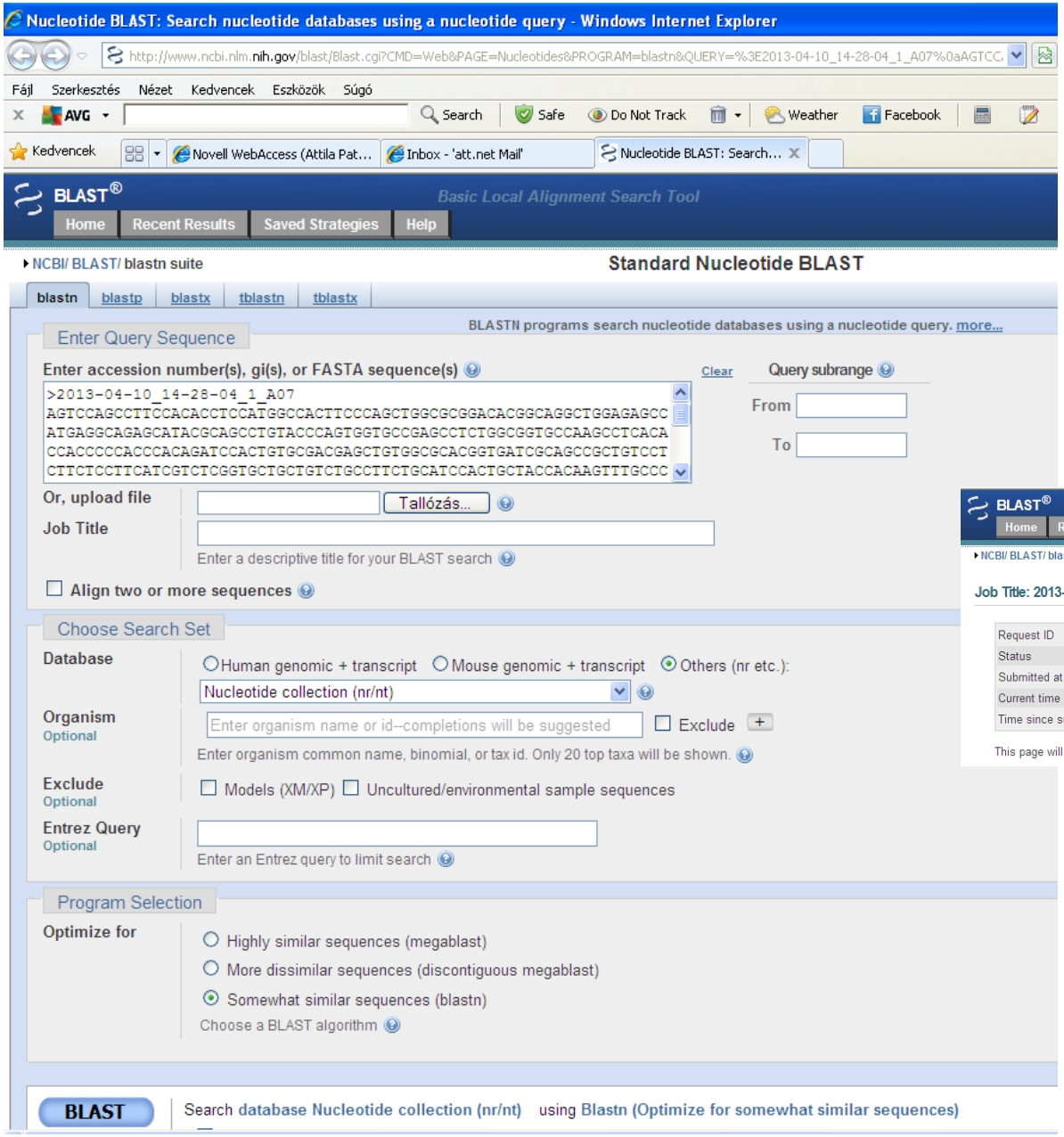


Wild type (normal seq)

Analysing sequencing data

Databases: Blast (Basic Local Alignment Search Tool)




Nucleotide BLAST: Search nucleotide databases using a nucleotide query - Windows Internet Explorer
http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE=Nucleotides&PROGRAM=blastn&QUERY=%3E2013-04-10_14-28-04_1_A07%0aAGTCC
Fájl Szerkesztés Nézet Kedvencek Eszközök Súgó
Search Safe Do Not Track Weather Facebook
Kedvencek
Novell WebAccess (Attila Pat...
Inbox - 'att.net Mail'
Nucleotide BLAST: Search...
BLAST®
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Recent Results
Saved Strategies
Help
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BLASTN programs search nucleotide databases using a nucleotide query. more...
Enter Query Sequence
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Query subrange
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To
>2013-04-10_14-28-04_1_A07
AGTCCAGCCTTCCACACCTCCATGGCCACTTCCCAGCTGGCGCGGACACGGCAGGCTGGAGAGCC
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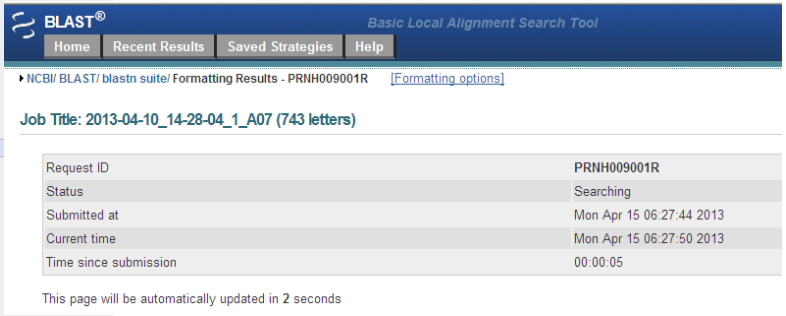
CTTCTCCTTCATCGTCTCGGTGCTGCTGTCTGCCTTCTGCATCCACTGCTACCACAAGTTTGCCC
Or, upload file
Tallózás...
Job Title
Enter a descriptive title for your BLAST search
Align two or more sequences
Choose Search Set
Database
Human genomic + transcript
Mouse genomic + transcript
Others (nr etc.):
Nucleotide collection (nr/nt)
Organism
Optional
Enter organism name or id--completions will be suggested
Exclude
Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.
Exclude
Optional
Models (XM/XP)
Uncultured/environmental sample sequences
Entrez Query
Optional
Enter an Entrez query to limit search
Program Selection
Optimize for
Highly similar sequences (megablast)
More dissimilar sequences (discontiguous megablast)
Somewhat similar sequences (blastn)
Choose a BLAST algorithm
BLAST
Search database Nucleotide collection (nr/nt) using Blastn (Optimize for somewhat similar sequences)


BLAST®
Basic Local Alignment Search Tool
Home
Recent Results
Saved Strategies
Help
NCBI/ BLAST/ blastn suite/ Formatting Results - PRNH009001R
[Formatting options]
Job Title: 2013-04-10_14-28-04_1_A07 (743 letters)
Request ID PRNH009001R
Status Searching
Submitted at Mon Apr 15 06:27:44 2013
Current time Mon Apr 15 06:27:50 2013
Time since submission 00:00:05
This page will be automatically updated in 2 seconds

Edit and Resubmit | Save Search Strategies | Formatting options | Download

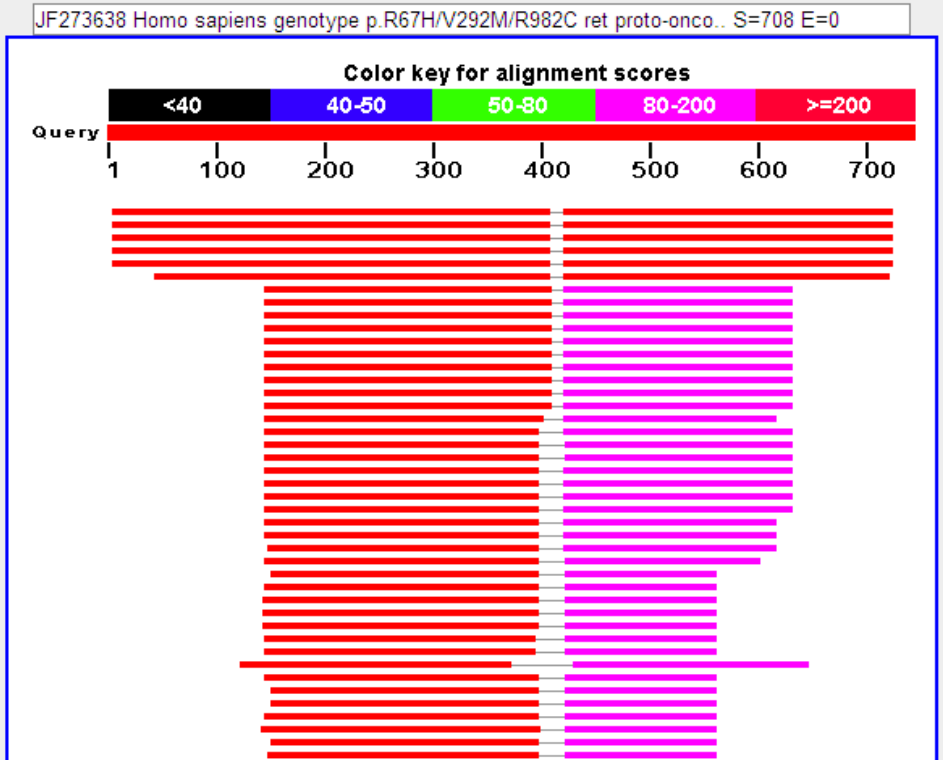
2013-04-10_14-28-04_1_A07 (743 letters)

Query ID	lcl|15547	**Database Name**	nr
Description	2013-04-10_14-28-04_1_A07	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.2.28+ Citation
Query Length	743		

Other reports: Search Summary [Taxonomy reports] [Distance tree of results]

Graphic Summary

Distribution of 164 Blast Hits on the Query Sequence



Analysing sequencing data

Download GenBank Graphics Sort by: E value

Homo sapiens ret proto-oncogene (RET), RefSeqGene on chromosome 10

Sequence ID: ref|NG_007489.1| Length: 60283 Number of Matches: 2

Range 1: 42271 to 42674 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
708 bits(784)	0.0	401/404(99%)	2/404(0%)	Plus/Plus

```
Query  7      GCCTTCC-ACACCTCCATGGCCACTTCCCAGCTGGCGCGGACACGGCAGGCTGGAGAGCC  65
              ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42271  GCCTTCCCACACCTCCATGGCCACTTCCCAGCTGGCGCGGACACGGCAGGCTGGAGAGCC  42330

Query  66     ATGAGGCAGAGCATACGCAGCCTGTACCCAGTGGTGCCGAGCCTCTGGCGGTGCCAAGCC  125
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Sbjct  42451  CCGCTGTCCTCTTCTCCTTCATCGTCTCG
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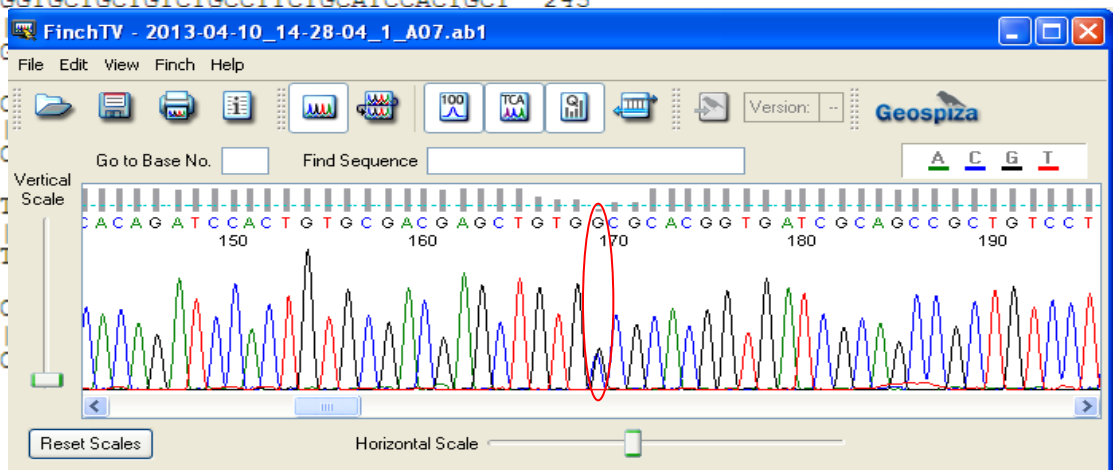
TGC-TGG csere



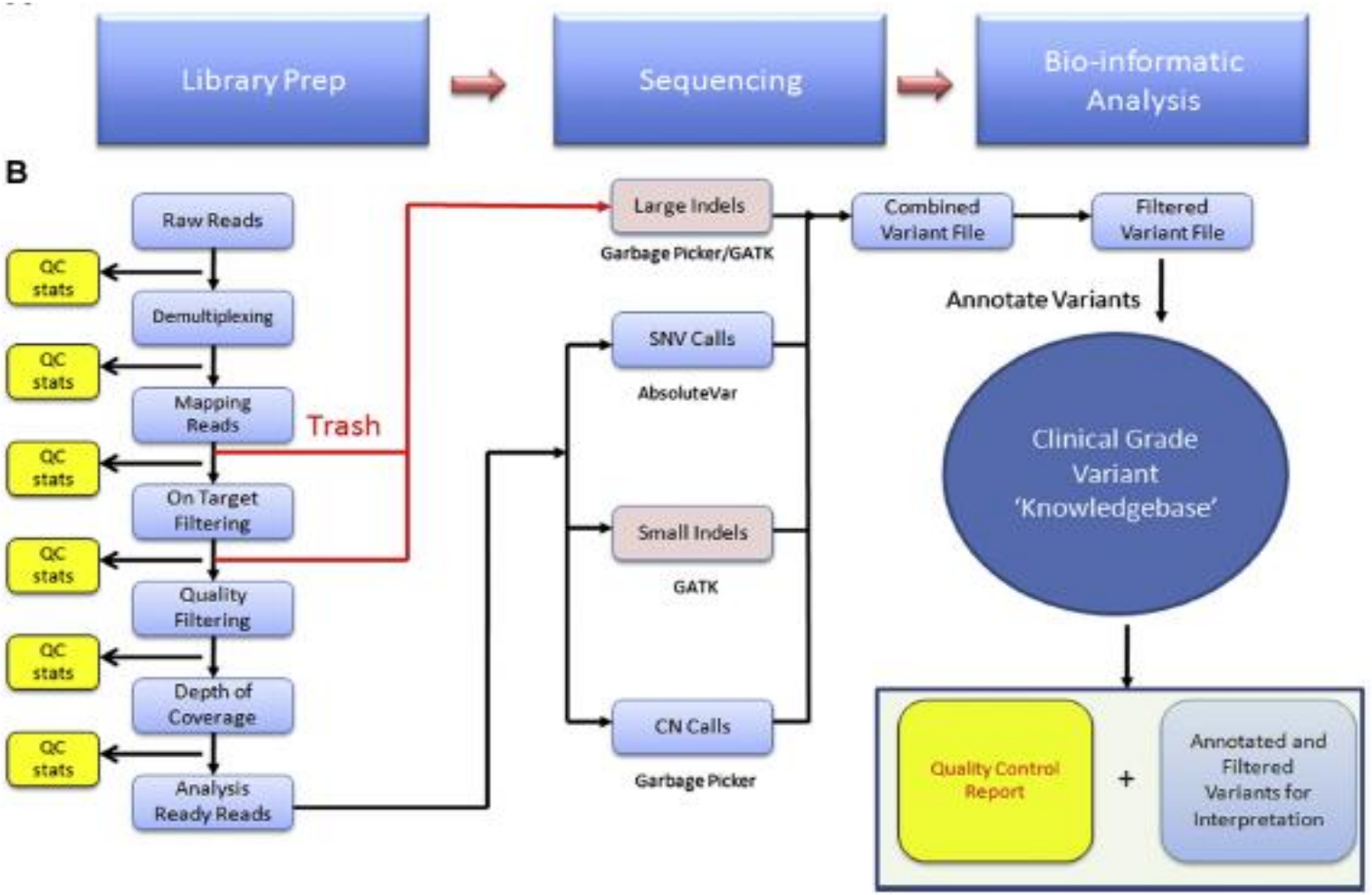
Next generation sequencing

Targeted sequencing:

gene panel (multigenic disorders, ie. oncology panel, pharmacogenomics panel…)

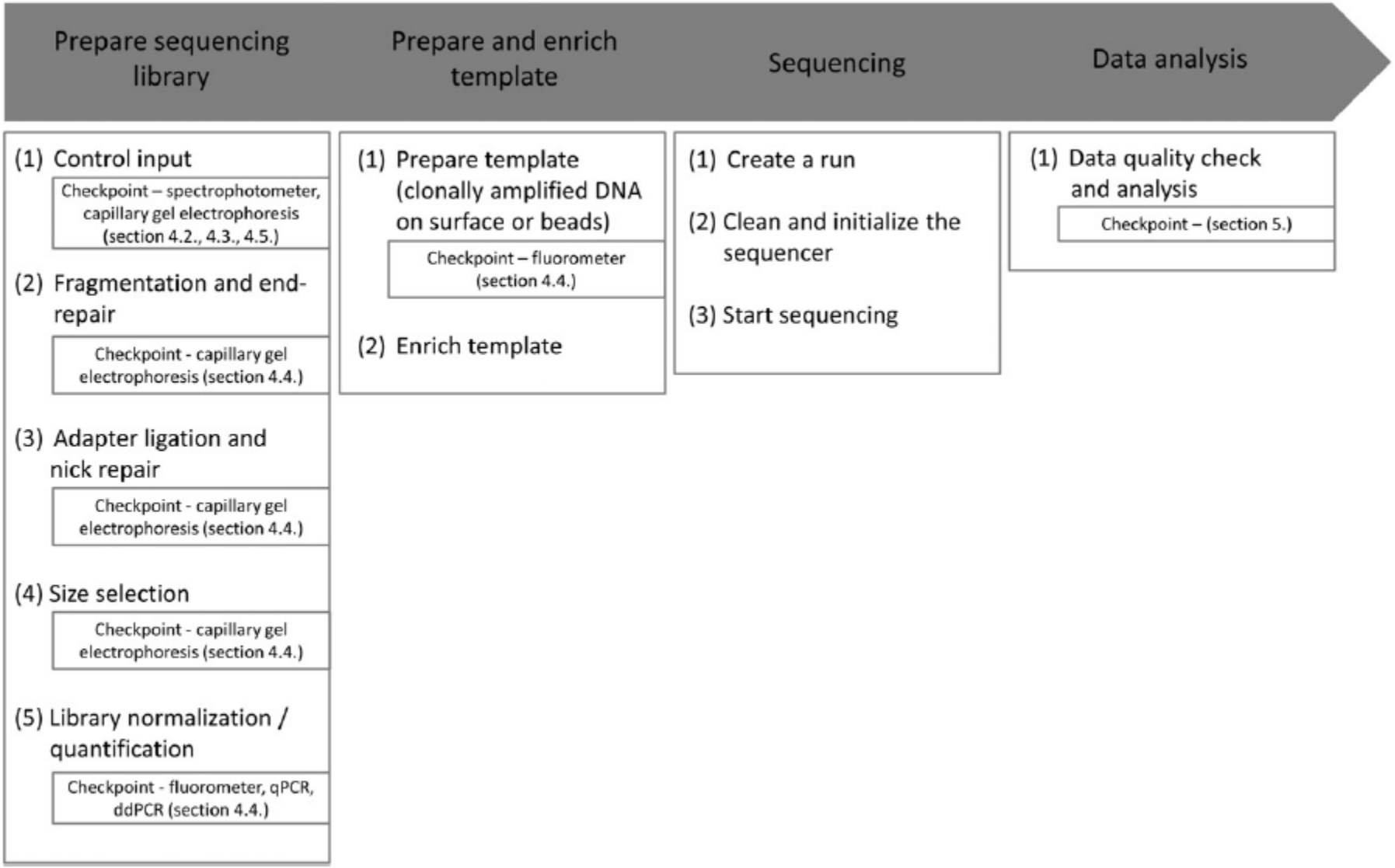
Exome sequencing: research and diagnosis

NGS quality control



Cancer Genetics 206 (2014) 441e448

NGS quality control



C. Endrullat et al. / Applied & Translational Genomics 10 (2016) 2–9

Available gene panels

TruSight Tumor 170
Comprehensive panel detects small variants, amplifications, and fusions that contribute to solid tumor progression

TruSight RNA Fusion
Targeting 507 fusion associated genes in cancer; detects known and novel fusion gene partners.

TruSight One
Targeting > 4,800 genes; enabling labs to expand and streamline their assay portfolio and sequencing portfolio

TruSight HLA
Accurate, unambiguous, phase-resolved HLA typing in a single assay

TruSight RNA Pan-Cancer
Targeting 1385 oncology genes for gene expression, variant and fusion detection in all RNA sample types including FFPE

TruSight Myeloid
Uses expert-defined content to identify somatic mutations in myeloid malignancies

TruSight Cancer
Targeting genes previously linked to a predisposition towards cancer

→ 94 gén és kb. 200 SNP

TruSight Tumor 15
Focused panel assesses common somatic variants in solid tumors

TruSight Cardio
Focusing on identifying inherited cardiac conditions

TruSight Inherited Disease
Focusing on severe, recessive pediatric onset diseases

→ 554 gén

Available gene panels

ClearSeq Comprehensive Cancer

This panel targets 151 key disease-associated genes that have been implicated in studies of a wide range of cancers (eg. breast, lung, colorectal, AML) and is designed for deep coverage of target bases for confident variant detection.
Compatible with SureSelect Target Enrichment System.

ClearSeq AML

Targets 20 genes found to be frequently mutated in acute myeloid leukemia (AML).This panel is designed for full coverage of target regions with multiple amplicons covering each target for greater confidence in somatic variant calling.
Compatible with HaloPlex^{HS} and HaloPlex Target Enrichment Systems.

ClearSeq Cancer

Identify somatic variants in 47 genes targeting known COSMIC hotspots found to be associated with a broad range of cancer types as well as published drug targets.
Compatible with HaloPlex^{HS} and HaloPlex Target Enrichment Systems.

ClearSeq Human DNA Kinome

This panel targets a comprehensive set of kinases and kinase related genes for enrichment, including over 500 kinases and 612 genes. This panel is compatible with post-capture (SureSelectXT) and pre-capture (SureSelectXT2) pooling – (XT2 - pool up to 16 samples per enrichment) available.
Compatible with SureSelect Target Enrichment System.

ClearSeq Human RNA Kinome

This panel targets a comprehensive set of kinases and kinase related transcripts for enrichment, including over 500 kinases and 612 genes.
Compatible with SureSelect Target Enrichment System.

Available gene panels
Roche-Nimblegen

Target Enrichment

Hybridization

- SeqCap Epi Choice Enrichment Kit
- SeqCap Epi CpGiant Enrichment Kit
- SeqCap EZ Developer
- SeqCap Epi Developer Enrichment Kits
- SeqCap EZ Exome UTR plus Kit
- SeqCap EZ Exome Plus Kits
- SeqCap EZ Exome v3.0 Kit
- SeqCap EZ MedExome Kit
- SeqCap RNA Choice
- SeqCap starter kits
- SeqCap EZ choiceXL
- SeqCap lncRNA
- SeqCap EZ HGSC VCRome
- SeqCap RNA Developer Enrichme Kit
- SeqCap EZ choice

Amplicon

- HEAT-Seq Ultra Choice Designs
- HEAT-Seq Choice Designs
- Heat-Seq Reagents

Target Enrichment Reagents and Accessories

- SeqCap EZ Developer Reagent
- SeqCap EZ Oligo Kit B
- SeqCap Adapter Kit A
- SeqCap Adapter Kit B
- SeqCap Accessory Kit V2
- SeqCap EZ Hybridization and Wash Kits
- SeqCap Pure Capture Bead Kit
- SeqCap EZ Oligo Kit A

Software

- SignalMapSoftware
- Nimble Design Software

Benefits of SeqCap EZ MedExome Kit

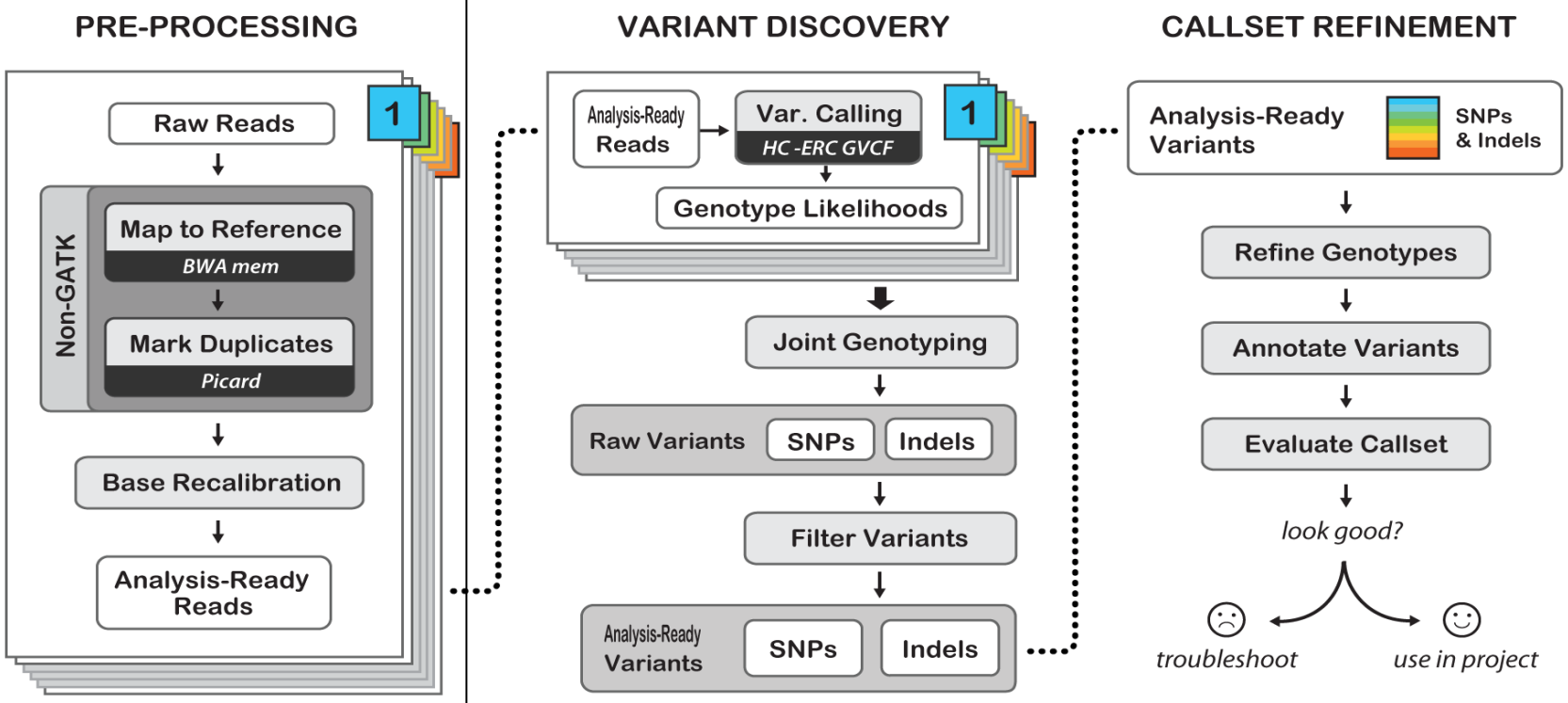
- >85% on-target rate with high uniformity across the targeted region*
- ~98% sensitivity for SNP detection and >99% specificity for SNP allele classification*
- Compatibility with the mitochondria-specific design to extend exome coverage
- Ability to add up to 200 Mb of user-defined custom regions to the existing MedExome design with the SeqCap EZ MedExome Plus configuration
- Combine with KAPA's library preparation products to create the HyperCap Workflow v2.0 for a fully integrated sample preparation solution.

Available gene panels

GeneReader-Qiagen, fully integrated platform

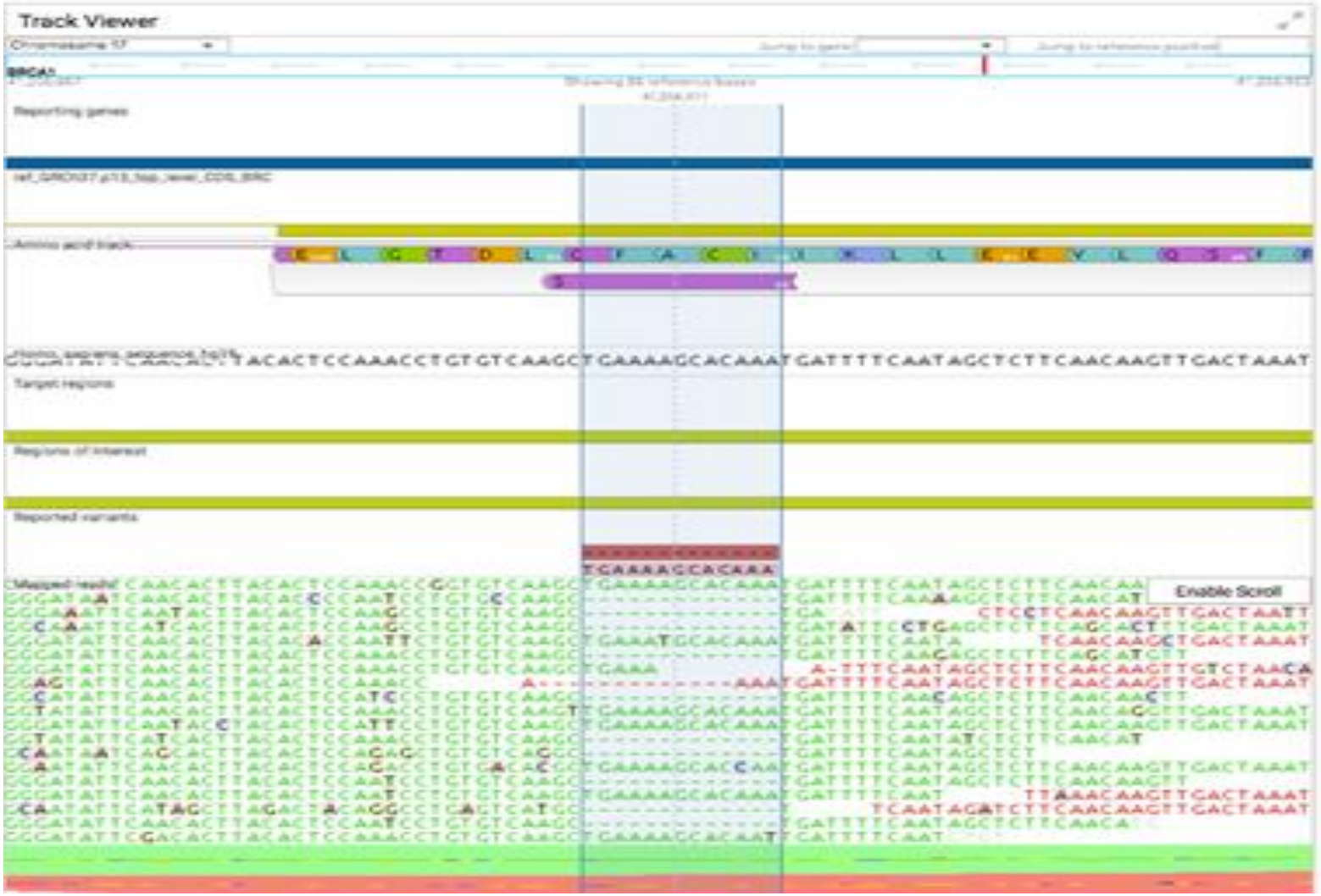
NGS: workflow

Bioinformatics: local



Best Practices for Germline SNPs and Indels in Whole Genomes and Exomes - June 2016

NGS sequencing of BRCA1/2 genes using GeneReader



Good coverage, long reads mapping to *BRCA1* gene

NGS sequencing of BRCA1/2 genes using GeneReader

QIAGEN Clinical Insight (QCI) Analysis report

BRCA 1/2 Report (Ready for Review)

1 Summary | 2 QC | 3 Variants | 4 Detailed QC | 5 History

3 Variants

Variants detected within regions of interest with more than significant coverage are found in 3.1 and variants with more than minimum coverage are found in 3.2. Regions of interest that could not be tested due to insufficient coverage are listed in table 3.3.
The coverage thresholds and minimum frequency cutoffs configured for the analysis workflow are listed in the History section.
Setting a variant review state to "Confirmed by review" moves it to 3.1, "Artifact" moves it to 3.2.
Only the variants in table 3.1 are exported as VCF and uploaded to QCI Interpret.

3.1 Reported variants

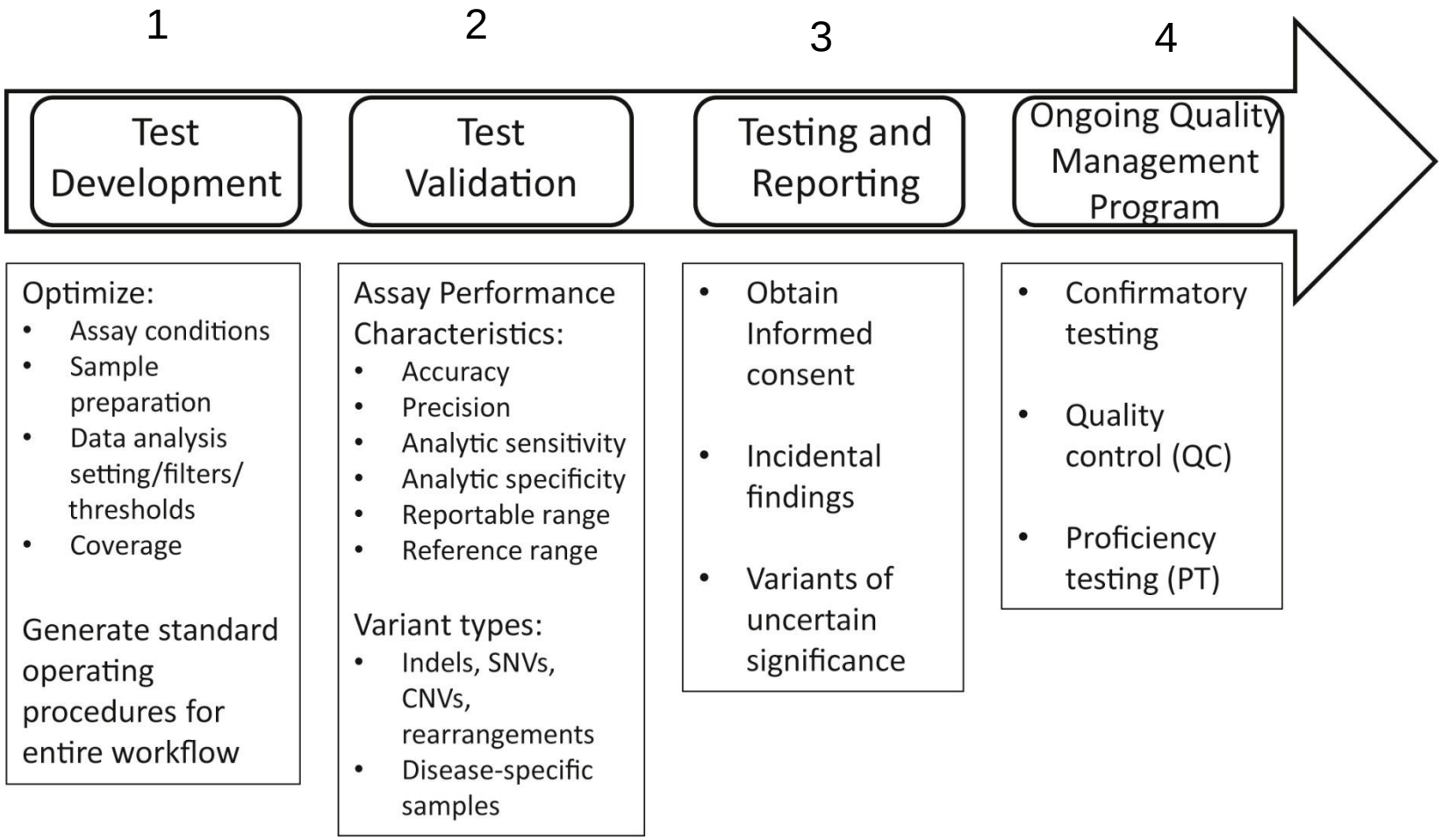
Variants that will be exported to VCF and uploaded to QCI Interpret. Initially contains: Variants detected within regions of interest with more than significant coverage and frequency above the cutoff set for the analysis workflow. These variants are assigned the initial review state "Valid".

15 variants

Default sorting | Filter table

Gene	Type	c. variant	p. variant	Impact	%	Qual	F/R test	Repeat	C-score	Count	F Count	R Count	Coverage	Region	Chr	ROI	Review	Comment	Edit
BRCA2	SNV	c.-26G>A			49.40	200	1.00	No	0.91	7,810	4,128	3,682	15,809	32890572	13	Yes	Valid		Edit
BRCA2	SNV	c.3396A>G			99.33	200	1.00	No	0.93	28,633	10,131	18,502	28,825	32911888	13	Yes	Valid		Edit
BRCA2	SNV	c.4563A>G			99.87	200	1.00	No	0.98	22,854	12,612	10,242	22,884	32913055	13	Yes	Valid		Edit
BRCA2	SNV	c.6513G>C			99.74	200	1.00	No	0.98	25,465	14,122	11,343	25,532	32915005	13	Yes	Valid		Edit
BRCA2	SNV	c.7242A>G			99.48	200	1.00	No	0.99	2,479	1,239	1,240	2,492	32929232	13	Yes	Valid		Edit
BRCA2	SNV	c.7397T>C	p.Val2466Ala	mis-sense	99.70	200	1.00	No	0.99	41,955	24,299	17,656	42,080	32929387	13	Yes	Valid		Edit
BRCA2	SNV	c.7806-14T>C			99.92	200	1.00	No	0.93	13,445	6,358	7,087	13,456	32936646	13	Yes	Valid		Edit
BRCA1	SNV	c.4837A>G	p.Ser1613Gly	mis-sense	47.50	200	1.00	No	0.89	18,233	9,450	8,783	38,387	41223094	17	Yes	Valid		Edit
BRCA1	SNV	c.4308T>C			50.64	200	1.00	No	0.95	14,417	8,735	5,682	28,468	41234470	17	Yes	Valid		Edit
BRCA1	SNV	c.3548A>G	p.Lys1183Arg	mis-sense	50.62	200	1.00	No	0.99	5,364	2,993	2,371	10,596	41244000	17	Yes	Valid		Edit
BRCA1	SNV	c.3113A>G	p.Glu1038Gly	mis-sense	49.08	200	1.00	No	0.91	2,799	1,751	1,048	5,703	41244435	17	Yes	Valid		Edit
BRCA1	SNV	c.2612C>T	p.Pro871Leu	mis-sense	49.17	200	1.00	No	0.98	7,551	4,920	2,631	15,356	41244936	17	Yes	Valid		Edit
BRCA1	SNV	c.2311T>C			49.57	200	1.00	No	0.96	2,511	1,357	1,154	5,066	41245237	17	Yes	Valid		Edit
BRCA1	SNV	c.2082C>T			50.19	200	1.00	No	0.95	4,719	1,976	2,743	9,403	41245466	17	Yes	Valid		Edit
BRCA1	Deletion	c.269_281delTTTGTGCTTTTCA	p.Ile90fs	frame-shift	53.68	200	1.00	No	0.98	13,058	7,320	5,738	24,325	41256905..41256917	17	Yes	Valid		Edit

Easy to use, all relevant interpretation included



The Journal of Molecular Diagnostics 2015 17, 472-482DOI: (10.1016/j.jmoldx.2015.06.001)

Clinical use of molecular biological techniques in diagnosis of hereditary endocrine syndromes

Diagnostics

Tumour syndromes:

Multiple Endocrine Neoplasia type 1 **MEN1**

Multiple Endocrine Neoplasia type 2 **MEN2**

Von Hippel-Lindau syndrome

Hereditary pheochromocytoma/paraganglioma syndrome

Other endocrine disorders:

21-hydroxylase deficiency

17alfa-hydroxylase/17,20-lyase deficiency

combined pituitary deficiency (mutation analysis of PROP1 gene)

Familiar hypocalcuric hypercalcemia (mutation analysis of CaSR gene)

Hereditary endocrine tumour syndromes and genes mutated in pheochromocytoma/paraganglioma

Syndrome	Gene	Identification
• Neurofibromatosis 1	NF1	(1990)
• vonHippel-Lindau	VHL	(1993)
• MEN2	Ret	(1994)
• PGL1	SDHD	(2000)
• PGL2	SDHAF2	(2010)
• PGL3	SDHC	(2001)
• PGL4	SDHB	(2000)
Others		
• Pheo, neuroblastoma, lung cc.	KIF1Bbeta	(2008)
• Paraganglioma, erythrocytosis	PHD2	(2008)
• Pheo, paraganglioma	TMEM127	(2010)
• Pheo, paraganglioma	SDHA	(2011)
• Pheo, paraganglioma	MAX	(2011)
• Pheo	FH	(2014)
• Pheo	MDH2	(2015)
• Pheo	GOT2	(2018)
• Pheo	SLC25A	(2018)

Common genetic feature: autosomal dominant inherited syndromes

Folllow-up of patients with germline patogenic mutations

Follow-up:

periodical screening (catecholamine metabolites, imaging…)

genetic counseling, mutation screening in first degree relatives

Mutation positive

Mutation negative: nothing to do

Genetic analysis in Pheo/PGL

- the “gold standard” methodology
  - PCR amplification of coding region of target genes followed by Sanger sequencing.
  - For large deletion analysis multiple ligation probe amplication (MLPA)
  - The Endocrine Society clinical practice guideline recommend the use of a clinical feature-driven diagnostic algorithm to establish the priorities for specific genetic testing in Pheo/PGL patients with suspected germline mutations delivered within the framework of health care

J Clin Endocrinol Metab, June 2014, 99(6):1915–1942

Genetic testing for Diagnosis of Pheochromocytoma and Paraganglioma (PPGL) Endocrine Society Guideline

- 3.1We recommend that **all patients with PPGLs should be engaged in shared decision making for genetic testing**.(**1**QQQE)

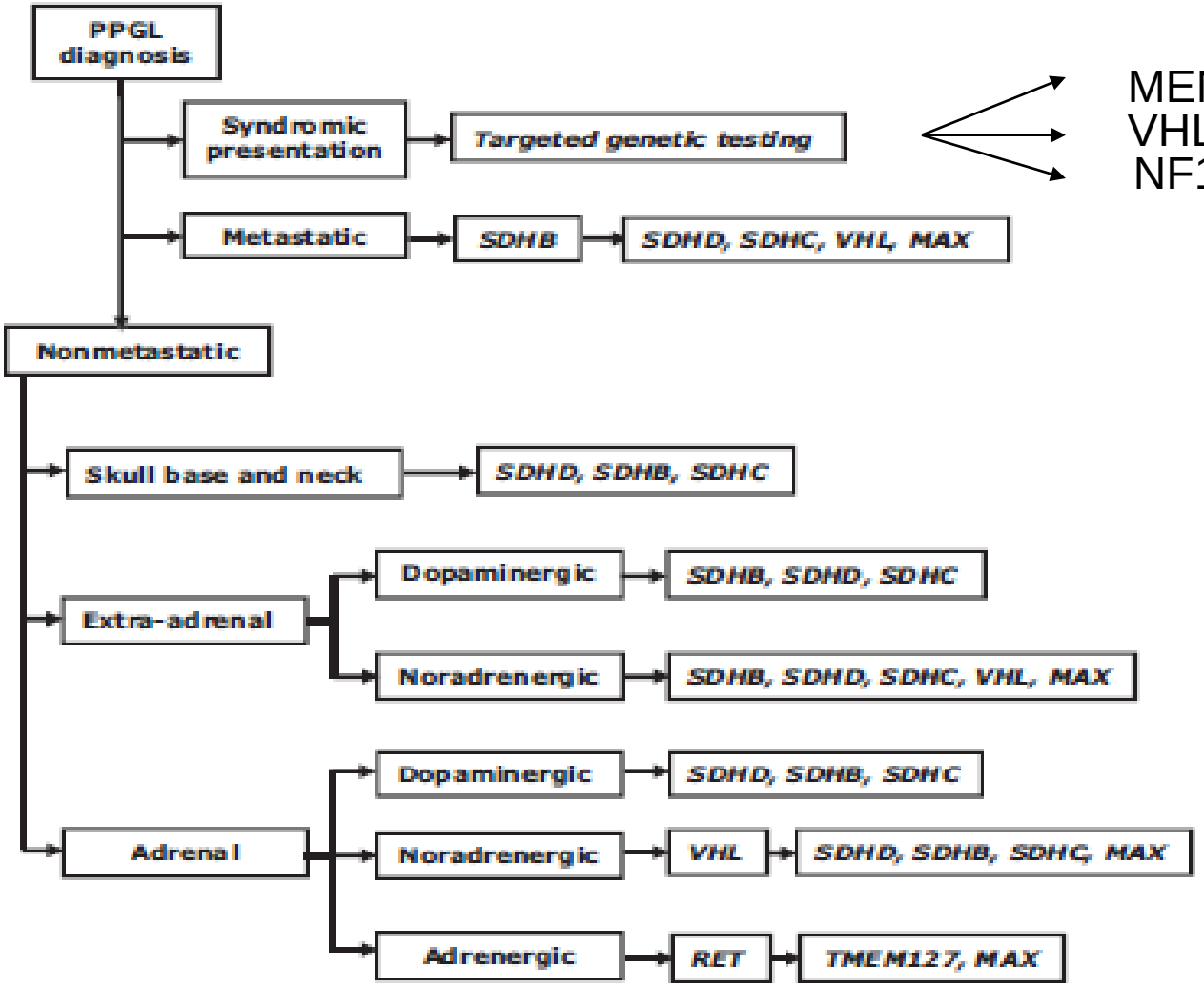
- 3.2 We recommend the use of a **clinical feature-driven diagnostic algorithm** to establish the priorities for specific genetic testing in PPGL patients with suspected germline mutations. (**1**QQQE)

- 3.3 We suggest that patients with paraganglioma undergo testing of succinate dehydrogenase (SDH) mutations and that patients with metastatic disease undergo testing for *SDHB* mutations. (**2**QQQE)

- 3.4 We recommend that genetic testing for PPGL be delivered within the framework of health care. Specifically, pretest and post-test counseling should be available. All tests for PPGL genetic testing should be performed by accredited laboratories. (Ungraded recommendation)

J Clin Endocrinol Metab, June 2014, 99(6):1915–1942

Decisional algorithm for genetic testing in patients with a proven PGL



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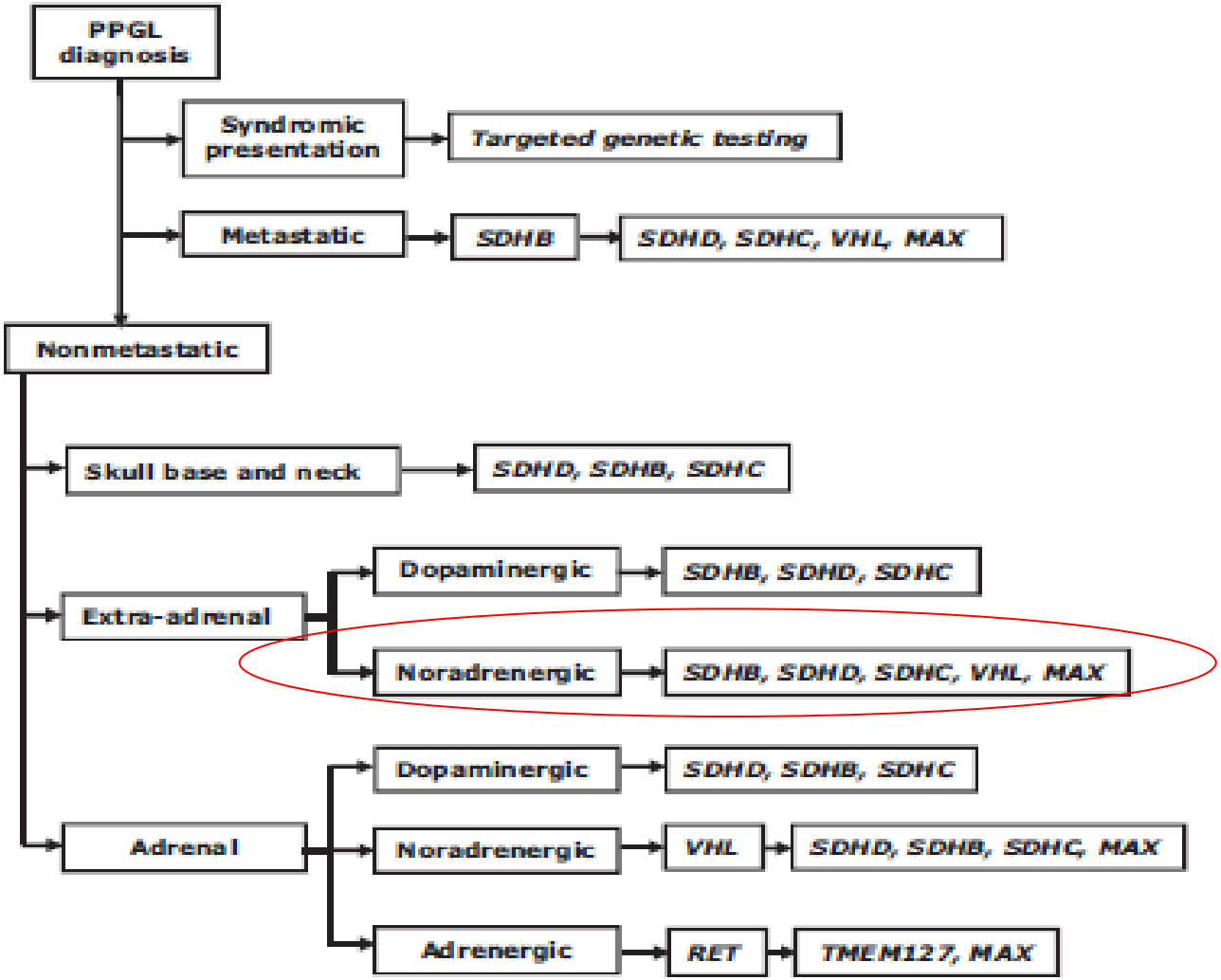


J Clin Endocrinol Metab, June 2014, 99(6):1915–1942

Case report

- Age: 33 years
- Symptoms: hypertension, eleveted heart rythm, sweating
- Family history: positive for hypertension
- Laboratory: once, elevated urinary catecholamine metabolite was measured
- Imaging: negative CT and MRI scans
    - Positive PET scan

- Diagnosis ???????

Could it be a familial syndrome?

Genetic test:

RET protooncogene:

pro: young, catecholamine secreting tumor

against: no MTC, no hyperparathyroidism

unlikely, to be a MEN2

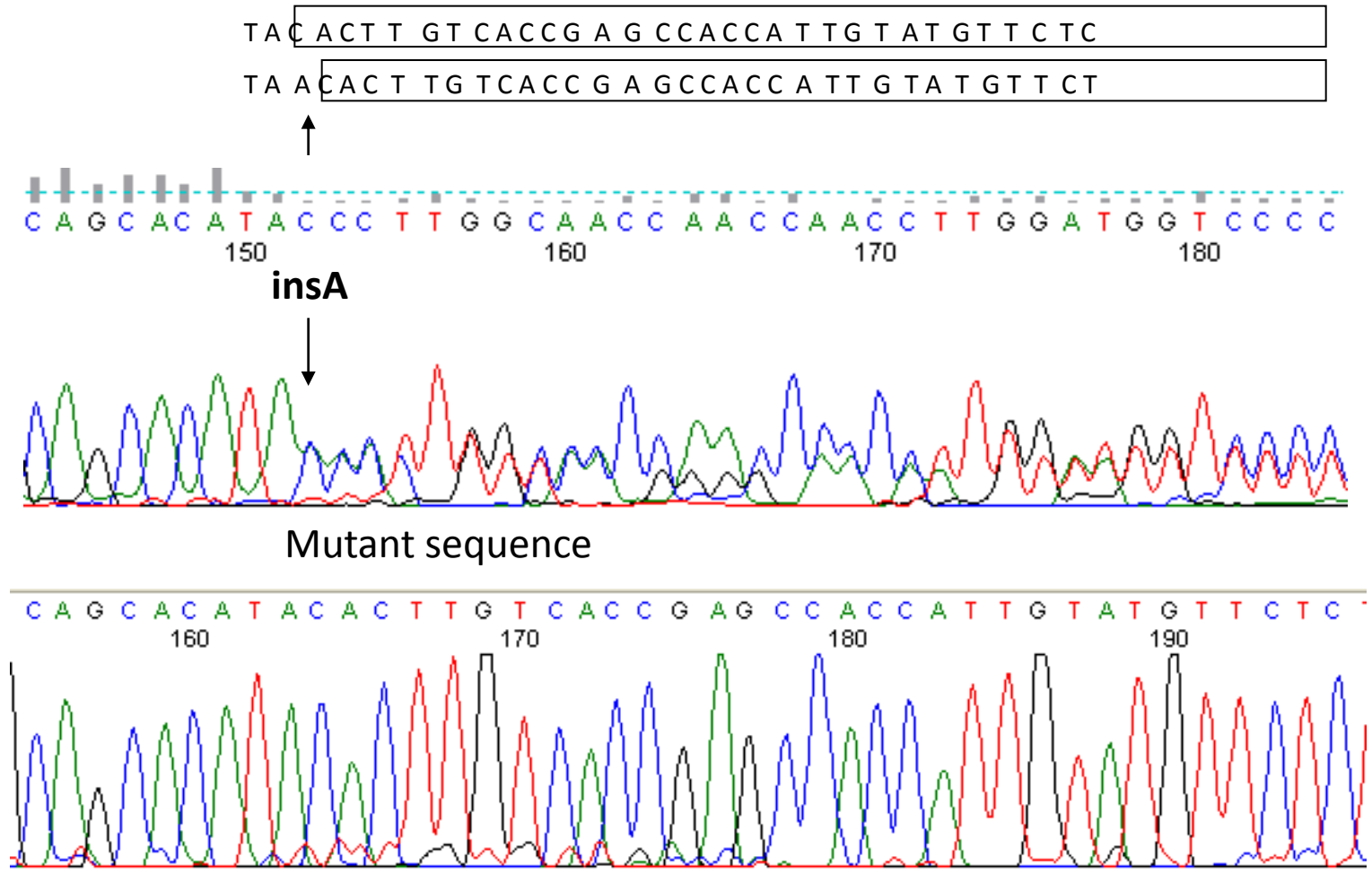
BUT

VHL: pro: young, catecholamine secreting tumor, could be VHL type2

SDHB, SDHD: only PGL/Pheo + family history

Definitive diagnosis: hereditary paraganglioma/pheochromocytoma syndrome

Frameshift (c.147-148 insA) SDHD mutation



Mutant sequence

Wild type sequence

Next generation sequencing based methods help in genetic diagnosis of Pheo/PGL

Objective: a valid diagnostic test is needed

- 15 genes should be tested

(RET, VHL, SDHA, SDHAF2, SDHB, SDHC, SDHD, NF1, MAX, TMEM127, FH, MDH2, KIF1B, PHD2, EPAS)

- Significant work load and cost by Sanger sequencing (in most centers the testing of RET, VHL, SDHB and SDHD is approx. 2700 USD)

NGS-based strategies

Targeted sequencing

Exome sequencing

NGS in clinical diagnostic applications

Benefits:

using targeted next generation sequencing a **70% cost reduction and 66% increase in diagnostic yield compared** to Sanger sequencing can be achieved (Rattenberry E, et al. J Clin Endocrinol Metab. 2013; 10:10.)

Problems:

Library preparation

Bioinformatics

- basecalling algorithms performs similarly across different technologies (Gargis AS,et al. Nat Biotechnol. 2012; 30(11):1033–6. PMID: 23138292

- validation of multiplexed targeted enrichment and bioinformatic processing are needed in diagnostic application

Current guidelines for the diagnostic use of next generation sequencing state that the **validity of the selected bioinformatic software needs to be ensured by the local investigator**

Targeted sequencing using Truseq and Illumina (Swedish study-2015)

Library prep: Truseq custom amplicon enrichment

Sequencing: Illumina MiSEQ instrument

Bioinformatics: 3 different algorithms

1: MiSEQ Reporter, fully automatized and integrated software,
2: CLC Genomics Workbench, graphical interface based software, also commercially available
3: an inhouse scripted custom bioinformatic tool

Crona et al. Bioinformatic Challenges in Clinical Diagnostic Application of Targeted Next Generation Sequencing: Experience from Pheochromocytoma PLOS ONE | DOI:10.1371/journal.pone.0133210 July 31, 2015

NGS in clinical diagnostic applications

Analytical sensitivity

- 98.7% (1 of 77 unique variants was not deteced) – amplicon-based method run on Roche GS Junior (Rattenberry et al)

- 82.9-100% (depending on bioinformatics)- Illumina Miseq platform

Specificity

- Roche GS Junior-

  False variant calls due to homopolymer tracts resulted in numerous false-positive calls for every DNA sample (of 164 unique variants- 46 were probably homopolymer-related artifacts)
  Including 2 filtering steps:
  (1) evaluating only the coding sequence ± 5 bp (with the exception of 5′ and 3′ untranslated regions) resulted in only 4 probable artifacts
  (2) Using only a calls within 2 SDs of the mean for that variant for the patients on that run. Resulted in no false positivity.

- Illumina MiSeq

  >99.9 %

Targeted sequencing using Truseq and Illumina-Swedish study-2015

Evaluation of bioinformatical algorithms

	Mapped reads to HG19 reference, mean % (range)	Mapped reads to targeted regions, mean % (range)	Read coverage, mean (Range)	% X-fold coverage at targeted regions		
				1X, mean (range)	10X, mean (range)	30X, mean (range)
MiSEQ Reporter 2.1.43						
Run01	89.4 (82–92.2)	54 (48.3–64.1)	414 (284–565)	98.1 (97.6–98.4)	97.6 (97.4–98)	95.9 (94.6–96.9)
Run02	86.3 (82.1–88.5)	51.7 (46.6–61.4)	407 (283–571)	98.2 (97.9–98.5)	97.6 (97.3–98.3)	96.2 (94.6–97.3)
CLC Genomics Workbench 5.51						
Run01	86.9 (82.6–92)	56.9 (50.5–67.2)	552 (377–738)	98.4 (98–98.7)	97.9 (97.7–98.4)	97,6 (97,3–97,7)
Run02	84.1 (80–89.1)	55.1 (49.3–64.7)	526 (357–735)	98.5 (98–98.7)	97.8 (97.6–98.7)	97,6 (97,2–98)
In-house custom pipeline						
Run01	89.6 (82–92.2)	58 (51.5–68)	519 (357–699)	98.4 (98–98.7)	98 (97.9–98.3)	97 (96.2–97.6)
Run02	89.4 (82.3–92.4)	56.9 (50.4–66.1)	517 (355–711)	98.5 (98.2–98.7)	98.1 (97.8–98.5)	97.3 (96.3–98)

Results from read mapping. Data presented from three bioinformatics workflows; MiSEQ Reporter 2.1.43 (Smith Waterman algorithm mapper), CLC Genomics Workbench 5.51 (default CLC mapper) and in-house custom pipeline (Burrows Wheeler Alignment tool).

Targeted sequencing using Truseq and Illumina-Swedish study-2015

Evaluation of performance of bioinformatical algorithms

	True pos, *n*	True neg, *n*	False pos, *n*	False neg, *n*	Sensitivity, %	Specificity, %
MiSEQ Reporter 2.1.43						
Run01	47	119197	99	0	100	>99.99
Run02	47	119195	101	0	100	>99.99
Filtered, merged Runs 01+02	47	119190	94	0	100	>99.99
CLC Genomics Workbench 5.51						
Run01	39	119296	0	8	82.9	100
Run02	40	119296	0	7	85.1	100
Filtered, merged Runs 01+02	36	119296	0	11	76.7	100
In-house Custom pipeline						
Run01	42	119284	7	5	89.4	>99.99
Run02	43	119288	4	4	91.4	>99.99
Filtered, merged Runs 01+02	42	119285	4	7	87.2	>99.99

Sensitivity of targeted next generation sequencing compared to current golden standard (automated Sanger sequencing) covering 5683 basepairs. Results are presented separately for both sequencing runs as well. Filtered and merged results incudes only variants available in both sequencing runs. Pos; positive, neg; negative.

Only MiSEQ reporter identified all pathogenic variants in both sequencing runs

Exome sequencing in genetic diagnosis of Pheo/PGL

- **Exome sequencing** was succesfully used for **identification of novel susceptibility genes for Pheo/PGL**
  - MAX (Comino-Méndez I et al. 2011)
  - MDH2 (Cascon et al. 2015)
  - ATRX –somatic (Fishbein et al. 2015)

It was successfully tested in genetic testing of s**hort-rib thoracic dystrophies (McInerney-Leo AM** et al. 2013)

NO study evaluated systhematically the analytical performance of WES in Pheo/PGL

Prevalence of disease-causing mutations in Hungarian patients with Pheo/PGL syndrome (1998-2015)

Cause of Pheo/PGL	Age (years)	Malignant	Bilateral or multiple locations
Genetic cause (n=22)	34,6 (13-62)	6/21 (28.5 %)	**11/22 (50%)**
RET (n=4)	33,5 (23-45)	1/4 (25%)	2/4 (50 %)
VHL (n=4)	36 (13-55)	2/4 (50%)	1/4 (25 %)
SDHD (n=2)	23 (13-32)	0	1/2 (50 %)
SDHC (n=1)	62	-	-
SDHB (n=6)	30.3 (19-37)	**3/6 (50%)**	4/6 (75 %)
TMEM (n=3)	40 (22-51)	0/3 (0 %)	2/3 (66 %)
NF1 (n=2)	31	0/2 (0%)	0/2 (0%)
No genetic cause (n=71)	40,4 (13-78)	12/71 (16.6 %)	**3/71 (0,4%)**
Total (n=93)	**38,8 (13-78)**	**17/93 (18,2%)**	**14/93 (15%)**

Disease causing mutations identified among Hungarian patients with Pheo/PGL using Sanger sequencing

Case	Age	Manifestation	Gene/Mutation
1	35	Pheochromocytoma (recidive)	RET: Cys634Trp
2	45	Pheochromocytoma (bilateral)	RET: Cys611Tyr
3	31	Pheochromocytoma and later medullary thyroid carcinoma	RET: Cys634Trp
4	23	Pheochromocytoma (bilateral)	RET: Cys634Tyr
5	13	Pheochromocytoma (malignant, bilateral)	VHL: Arg79Gly *
6	55	Pheochromocytoma	VHL: Tyr156Cys
7	25	Pheochromocytoma	VHL: Arg167Gln
8	50	Pheochromocytoma	VHL: Leu63Pro
9	31	Pheochromocytoma	NF1**
10	31	Pheochromocytoma	NF1**
11	33	Paraganglioma (intrabdominal+head/neck, malignant)	SDHB:Cys253Tyr
12	32	Paraganglioma (intrabdominal+head/neck, malignant)	SDHB: Cys196Gly *
13	30	Paraganglioma intraabdominalis	SDHB: Cys243Tyr*
14	19	Pheochromocytoma + renal cell carcinoma	SDHB: Gly203Stop*
15	37	Paraganglioma (head/neck)	SDHB: c286+1G/A,
16	24	Paraganglioma intraabdominal	SDHB: Arg217Cys
17	62	Paraganglioma (head/neck)	SDHC: ivs+1G/T
18	32	Paraganglioma (intrabdominal+head/neck)	SDHD: c.147-148 insA
19	13	Pheochromocytoma	SDHD: His50Arg
20	51	Pheochromocytoma (bilateral) Paraganglioma (intraabdominalis and head/neck)	TMEM127: Leu155Stop*
21	22	Pheochromocytoma unilateral	TMEM127: Cys140Tyr
22	47	Pheochromocytoma bilateral	TMEM127: c572delC

Comparison of performance of exome sequencing to Sanger sequencing using Hungarian patients with SDHx germline mutations

Two platforms (one using Illumina and another one Complete Genomics) were tested. WES was performed by BGI Hong Kong

	Illumina	**Complete Genomics**
Library preparation	SureSelect Biotinylated RNA Library Agilent 51M	BGI 59Mb exome kit
Sequencing platform	Hiseq 2000	Complete genomics
Base calling	Illumina base calling Software 1.7	Complete genomics RTA 1.7 software
Alignment	Burrows-Wheeler Aligner (BWA	Terramap
SNP calling and annotation	GATK workflow (Samtools, Annovar)	GATK workflow (Samtools, Annovar)

Comparison of performance of exome sequencing to Sanger sequencing using Hungarian patients with SDHx germline mutations

Methods 2_variant assessment

Data used: Illumina only those variants which had a coverage >10 reads
Complete Genomics > 5 reads.

Variant assessment: SNPEFFECT (http://snpeffect.switchlab.org/about, version 3.4)

Allele frequencies and phenotype associations:

- dbSNP, http://www.ncbi.nih.gov/SNP
- National Comprehensive Cancer Network, http://www.nccn.org
- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
- SDHx mutation database, http://chromium.liacs.nl/lovd_sdh/
- NHLBI Exome Sequencing Project (ESP) Exome Variant Server http://evs.gs.washington.edu/EVS/

Variant assessment: pathogenity:

- Polyphen and SIFT

Comparison of performance of exome sequencing to Sanger sequencing using Hungarian patients with SDHx germline mutations

Results: CG versus Illumina

	Complete Genomics						Illumina		
Samples	CG1-PB	CG2-BB	CG3-KP	CG4-KJ	CG5-VB	CG6-MF	Ilum-HUN1	Ilum-HUN2	Ilum HUN3
Total	94259	94825	90133	92723	92481	90462	39358	39731	39766
1000genome and dbsnp	86033	87807	82763	85258	85539	82796	37757	38050	38078
1000genome specific	309	279	290	370	287	322	45	35	46
dbSNP specific	2647	2853	2742	2727	2770	2815		1118,00	116,00
dbSNP rate (%)	94,80	95.61	94.87	94.89	95.49	94.64		98,58	98,56
Novel	5270	3886	4338	4368	3885	4529	469	528	526
Hom	32015	33181	30296	31725	32812	30393	15518	15305	15333
Het	62244	61644	59837	60998	59669	60069	23840	24426	24433
Synonymous	10769	10786	10699	10967	10652	10787	9143	9275	9237
Missense	10471	10384	10229	10420	10217	10620	8009	7927	8020
Stopgain	123	118	103	111	114	101	51	43	45
Stoploss	27	36	30	29	34	33	24	27	26
Exonic	21429	21376	21106	21563	21047	21580	17052	17101	17167
Splicing	129	128	114	126	118	116	44	53	54
NcRNA	698	692	718	717	707	712	1044	1049	1100
UTR5	1318	1373	1366	1410	1331	1378	1133	1138	1198
UTR3	2985	2980	2869	2934	2928	2783	1737	1717	1744
Intronic	64597	65026	61025	62835	63263	60910	16070	16377	16241
Upstream	1309	1345	1201	1279	1247	1260	326	367	357
Downstream	1509	1617	1458	1580	1574	1452	161	163	163
Intergenic	285	288	276	279	266	271	1574	1556	1537
SIFT	1880	1815	1808	1813	1811	1900	888	835	854

Distribution of variants identified by two exome sequencing platforms

	Complete genomics	Illumina	Difference
Clinically important	**32000**	**25214**	**25%**
Missense	10390	7985	30%
Stopgain	**112**	**46**	**140%**
Stoploss	32	26	23%
Exonic	21350	17107	23%
Splicing	**122**	**50**	**144%**
Located in regulatory domains	**71008**	**22251**	**322%**
NcRNA	707	1064	33%
UTR5	1363	1156	18%
UTR3	**2913**	**1733**	**68%**
Intronic	**62943**	**16229**	**287%**
Upstream	**1274**	**350**	**264%**
Downstream	**1532**	**162**	**845%**
Intergenic	278	1556	460%

Comparison of performance of exome sequencing to Sanger sequencing using Hungarian patients with SDHx germline mutations

Exome sequencing versus sanger sequencing

Patient ID	Result of Sanger sequencing, target for exome seqeuncing	NGS platform used	Exome sequencing		
			Mutation confirmed	Category	Read number (ratio and read number between alleles
1A	SDHB: C196Gly	Illumina	**Yes**	Moderate	51 (0,53: 27/24)
1 B (mother of 1.A)	SDHB: Cys196Gly	Illumina	**Yes**	Moderate	60 (0,55: 27/33)
1C (Father of 1.A)	SDHB wild type	Illumina	**No alteration in SDHx genes**		
2.	SDHB: Arg217Cys	Complete Genomics	**Yes**	Moderate	46 (0,52: 22/24)
3.	SDHB: Cys253Tyr	Complete Genomics	**Yes**	Moderate	40 (0,45: 22/18)
4.	SDHB: Cys243Tyr	Complete Genomics	**Yes**	Moderate	37 (0,37: 24/14)
5.	SDHB: c286+1G/A	Complete Genomics	**Yes**	High	24 (0,45: 13/11)
6.	SDHB: Gly203Stop*	Complete Genomics	**Yes**	STOP	24 (0,41: 14/10)
7.	SDHC: ivs+1G/T	Complete Genomics	**Yes**	High	40 (0,57: 17/23)
8.	SDHD: le49_His50fs/ c.147_148insA	Complete Genomics	**Yes**	High	30 (0,43: 13/17)
9.	SDHD: His50Arg	Complete Genomics	**Yes**	Moderate	25 (0.56: 14/11)

Both platforms correctly identified the known pathogenic mutations

Exome sequencing versus sanger sequencing 2.

What other alterations were revealed?

	Variant ID	Read numbers of mutant / wild type /total alleles	Sanger sequencing confirmed	Functional prediction	Minor allele frequency
EGLN1	ENST00000366641:p.X187X 231557073 insC	9/41/50	**Not confirmed false positive**	Probably deleterious	No data
	ENST00000366641:p.X187X 231557073 insC	8/27/35	**Not confirmed false positive**	Probably deleterious	No data
	Rs12097901 was not detected	**False negative**	rs12097901 (Cys127Ser)	Neutral	0.26
KIF1B	***Rs229788:*** ENST00000377081:p.Tyr1133Cys	71/81/152	Yes	Probably deleterious	0.039-0.06
	rs77172218 ENST00000377081:p.Val1600Met	68/78/146	Yes	Neutral	0.004-0.01
	rs148690591 c.*2T>C 3'UTR NM_015074.3:c.*2T>C, XM_005263433.1:c.*2T>C	105/98/203	Yes	Unknown	0.0012
RET	***rs17158558*** ENST00000355710 p.Arg982Cys	22/21/43	Yes	Probably deleterious	0.022
	rs1799939 ENST00000355710:p.Gly691Ser	In 2 cases	Yes	Neutral	0.1-0.15
NF1	ENST00000358273:p.Asp896Val	In 2 cases	Not tested	Neutral	No data
	rs2525574 ENST00000444181:p.*639Arg	In six cases	Not tested	Probably deleterious	0.37-0.44

Two rare variants with deleterious function were identified in SDHB carriers in genes already associated with Pheo/PGL

PHENOTYPE MODIFIERS????

What alterations identified during exome sequencing should be reported

Pheo/PGL genes

	Criteria:	*Clinical Utility*	*Clinical Validity*			*Unknown Clinical Implications*
Genes	Bins:	**Bin 1 Medically actionable incidental information**	**Bin 2A Low risk incidental information**	**Bin 2B Medium risk incidental information**	**Bin 2C High risk incidental information**	**Bin 3**
	Examples:	*BRCA1/2 MLH1, MSH2 FBN1 NF1*	PGx variants and common risk SNPs	*APOE* Carrier status for recessive Mendelian disorders	Huntington Prion diseases ALS (SOD1)	**All other loci**
	Estimated number of genes/loci:	10s	10s (eventually 100s – 1000s)	1000s	10s	~20,000
	Alleles that would be reportable (YES) or not reportable (NO) in a clinical context					
Variants	Known deleterious	YES	YES/NO [1]	YES/NO [1]	YES/NO [1]	N/A [2]
	Presumed deleterious	YES	N/A [3]	YES/NO [1]	YES/NO [1]	NO [4]
	VUS	NO	N/A [3]	NO	NO	NO [4]
	Presumed benign	NO	N/A [3]	NO	NO	NO
	Known benign	NO	NO	NO	NO	NO

N/A: not applicable; VUS: Variant of uncertain significance

[1] Reporting through decision making with an appropriate provider if elected by the patient.

[2] By definition, variants in genes with unknown implications could not be considered deleterious.

[3] By definition, SNPs or PGx variants will either be present or absent.

[4] Variants in genes with unknown clinical implications would not be reported; however, they may serve as an important substrate for research, potentially uncovering new disease genes.

Fig. 1. Proposed system for "binning" of incidental WGS results

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Summary

NGS based platforms can be used in genetic analysis of Pheo/PGL associated genes

Exome sequencing using both Complete Genomics and Illumina platforms correctly identified the known pathogenic mutations

Library preparation highly affects the quantity of data, CG exome kit resulted in more than 3 times higher amount of variants

A base call with a coverage of >10reads was successfully validated by Sanger sequencing

False positive cases can be found, however by filtering strategies the number can be limited

False negative calls are linked to GC-rich and copy number alteration regions

Novel variants with phenotype modifier role can be identified using exome sequencing

Re-evaluation of exome sequencing datasets may allow to identify retrospectively patients with genetic alterations