

illumina Webinar Series
クリニカルシーケンス

アンプリコンシーケンシングによる 低頻度モザイクGNAS変異の 高感度検出

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Menu

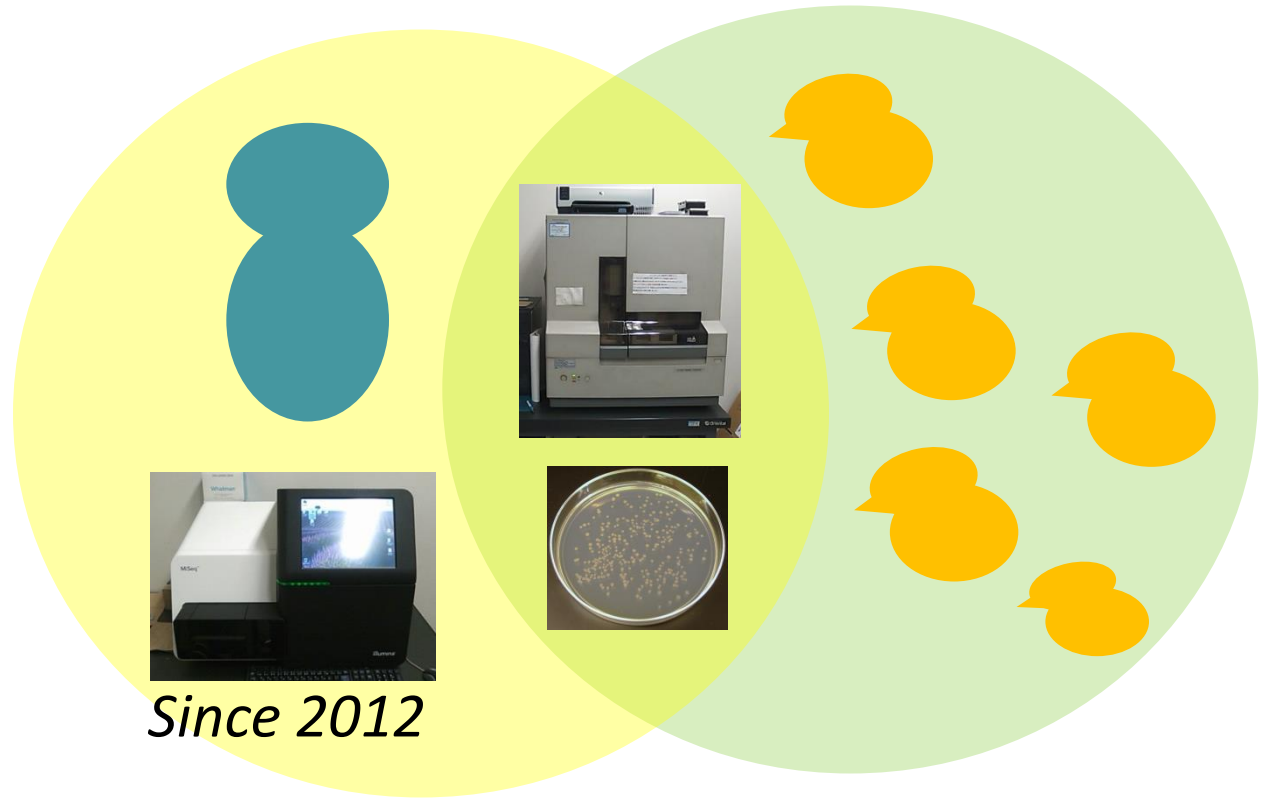
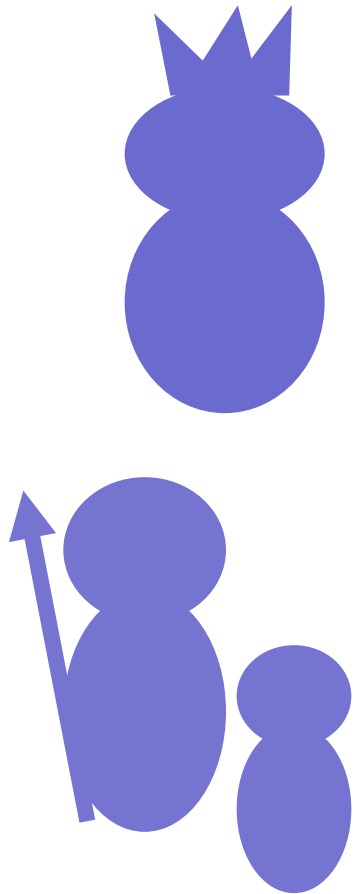
イントロダクション

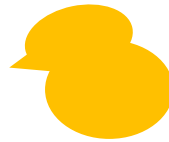
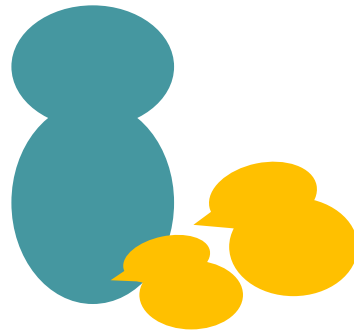
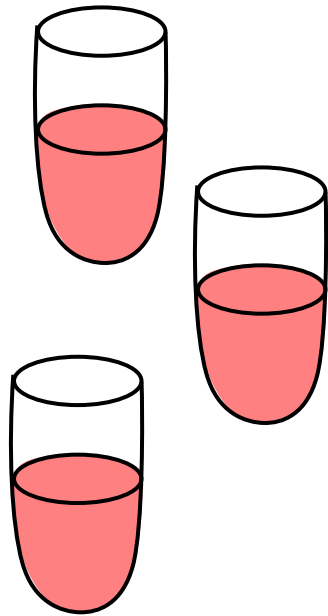
疾患について

ワークフロー

今後の展望





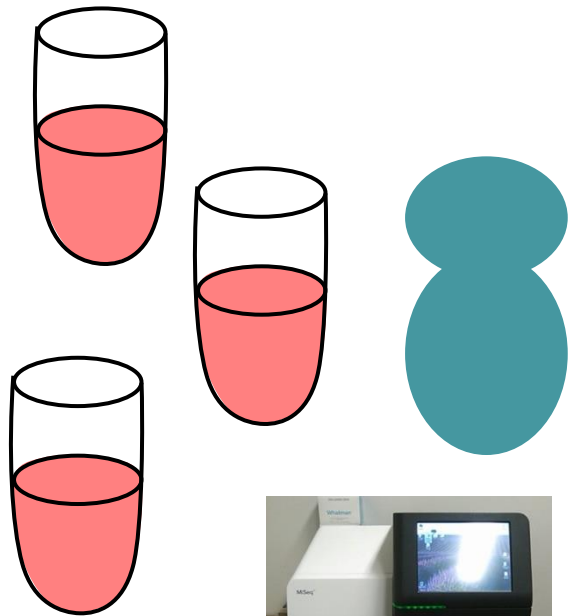


甲状腺担当

副肾担当

性腺担当

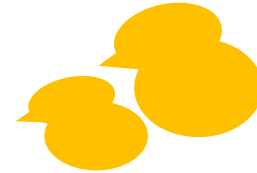
下垂体担当



Target
resequencing



甲状腺担当



副肾担当

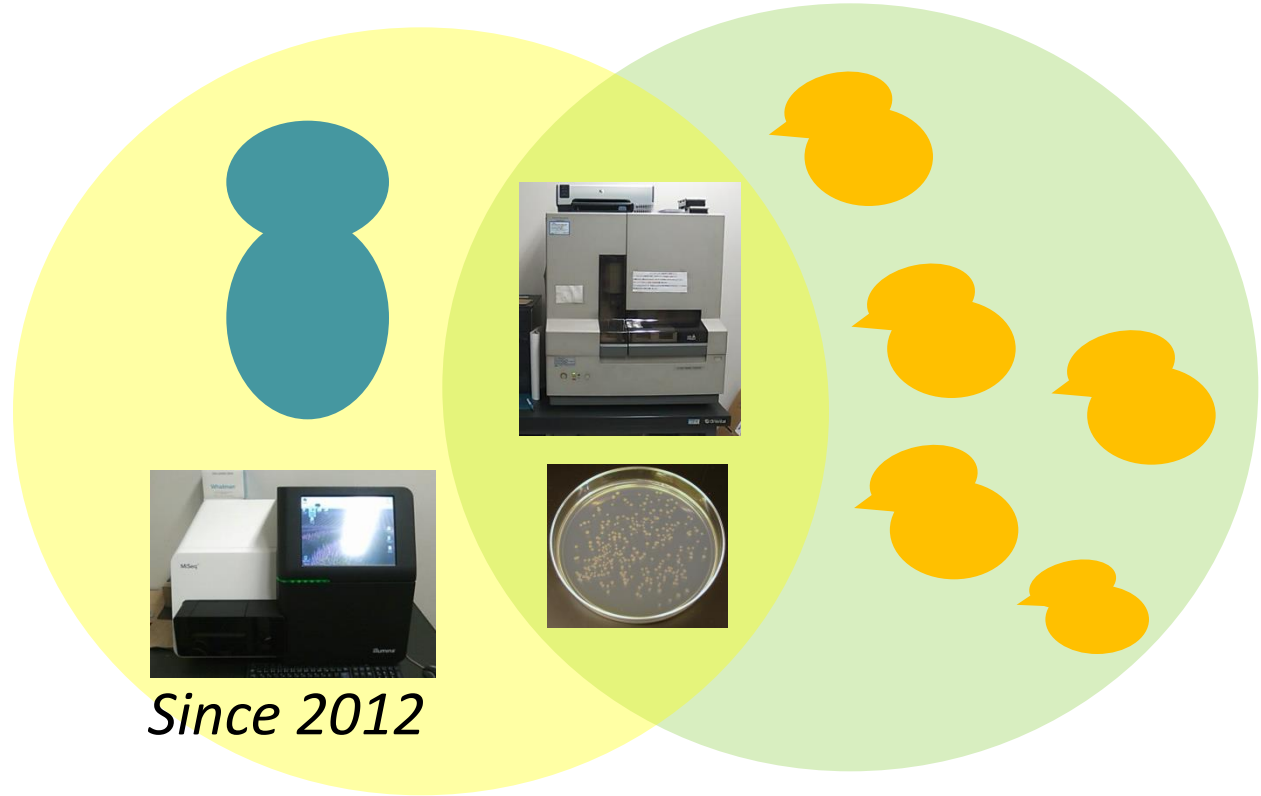
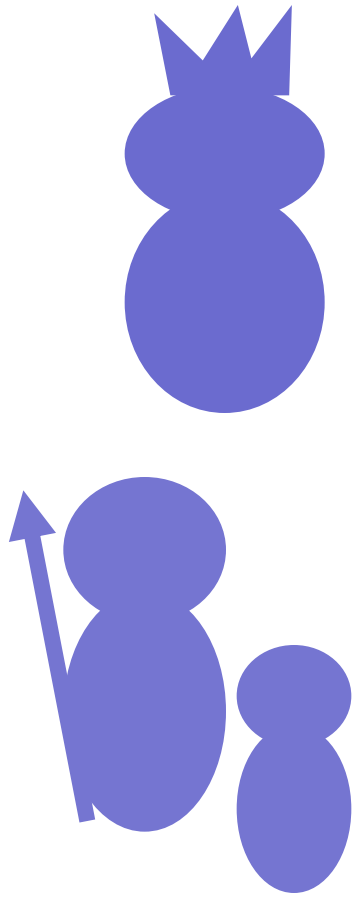


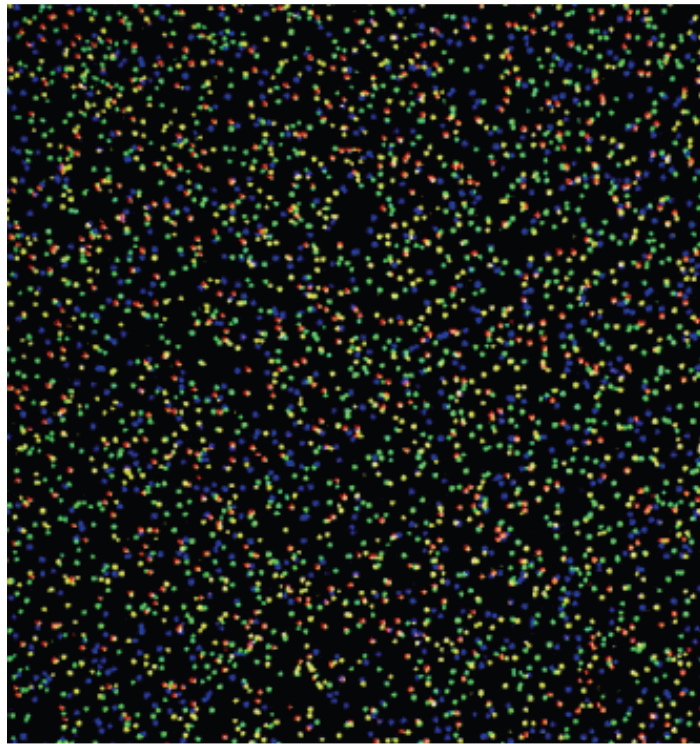
性腺担当



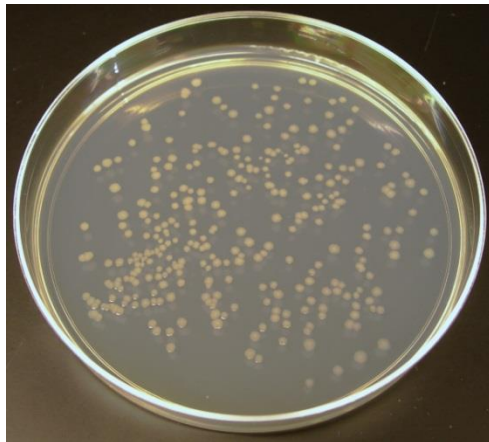
下垂体担当







2,000万クラスタ
12万円



100コロニー
3万円

Menu

イントロダクション

疾患について

ワークフロー

今後の展望



McCune-Albright症候群

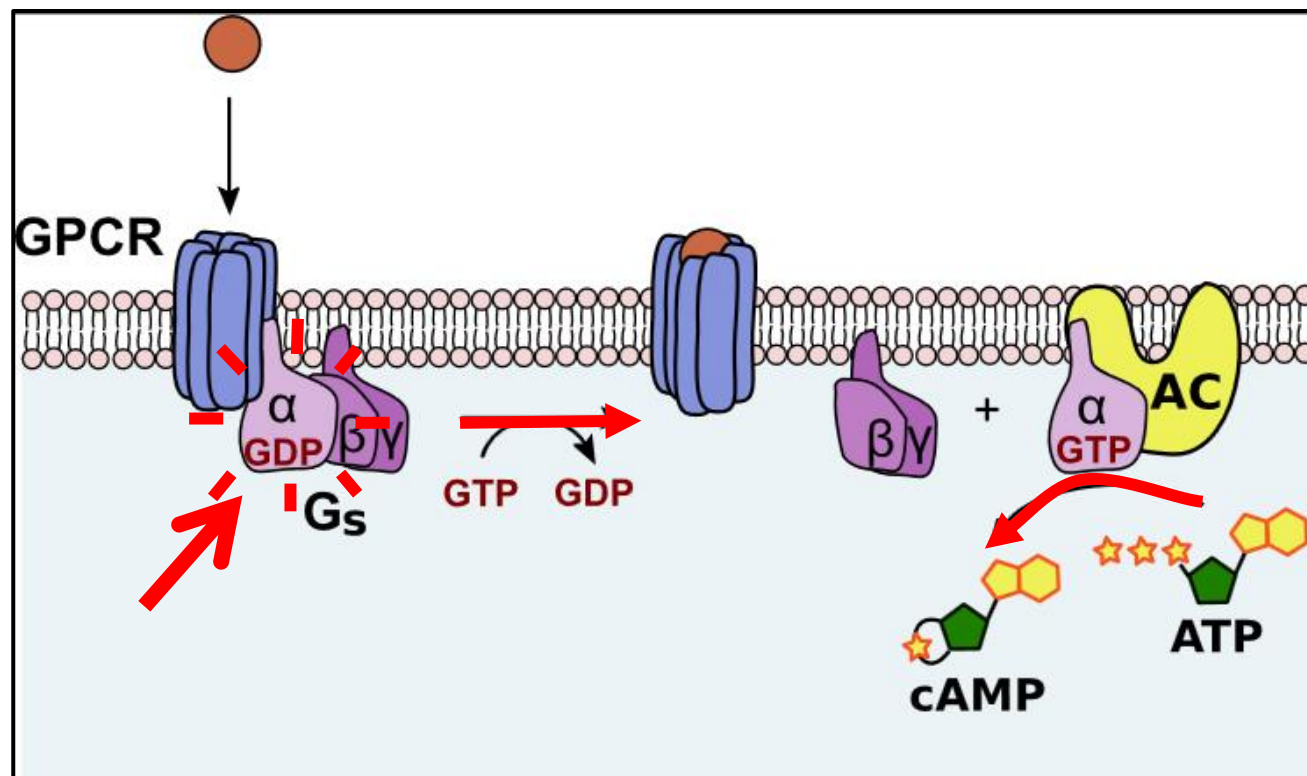
- * カフェオレ斑
線維性骨異形成
内分泌機能亢進
- * 機能亢進型*GNAS*変異
体細胞モザイク
- * 90%以上はArg201残基



<http://history.nih.gov/exhibits/rodbell/>

http://www.wheelsonline.com/ortho/fibrous_dysplasia

GNAS

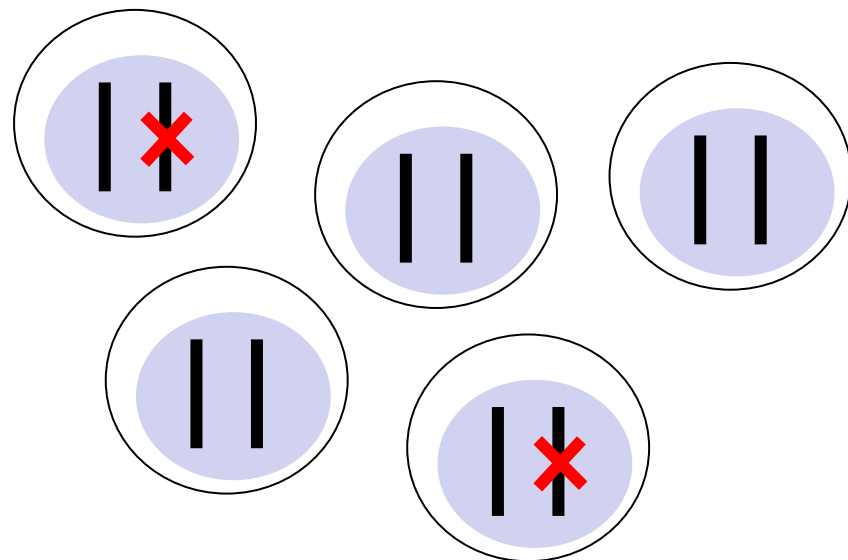


線維性骨異形成

内分泌機能亢進

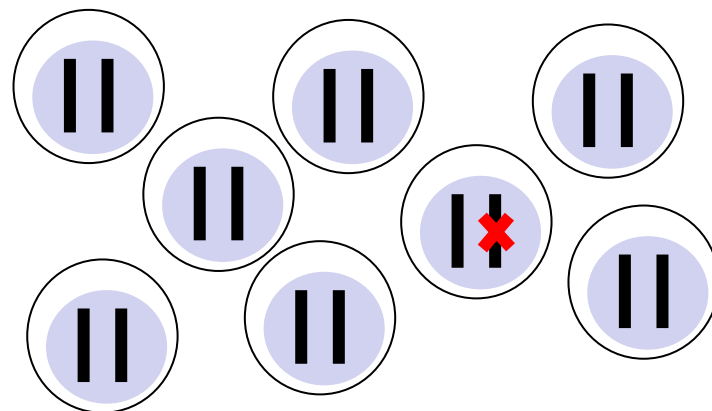
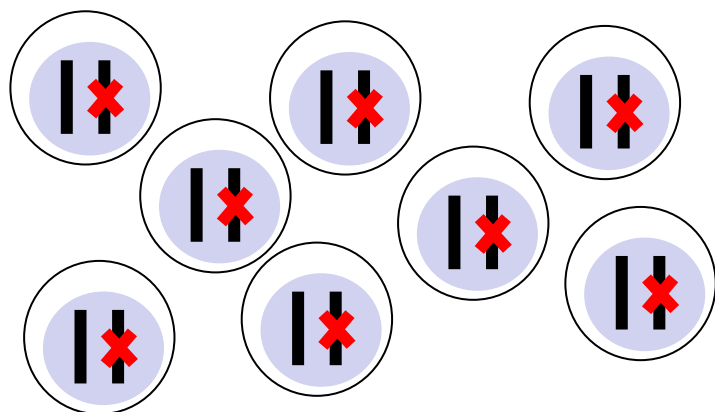
カフェオレ斑

体細胞モザイク変異



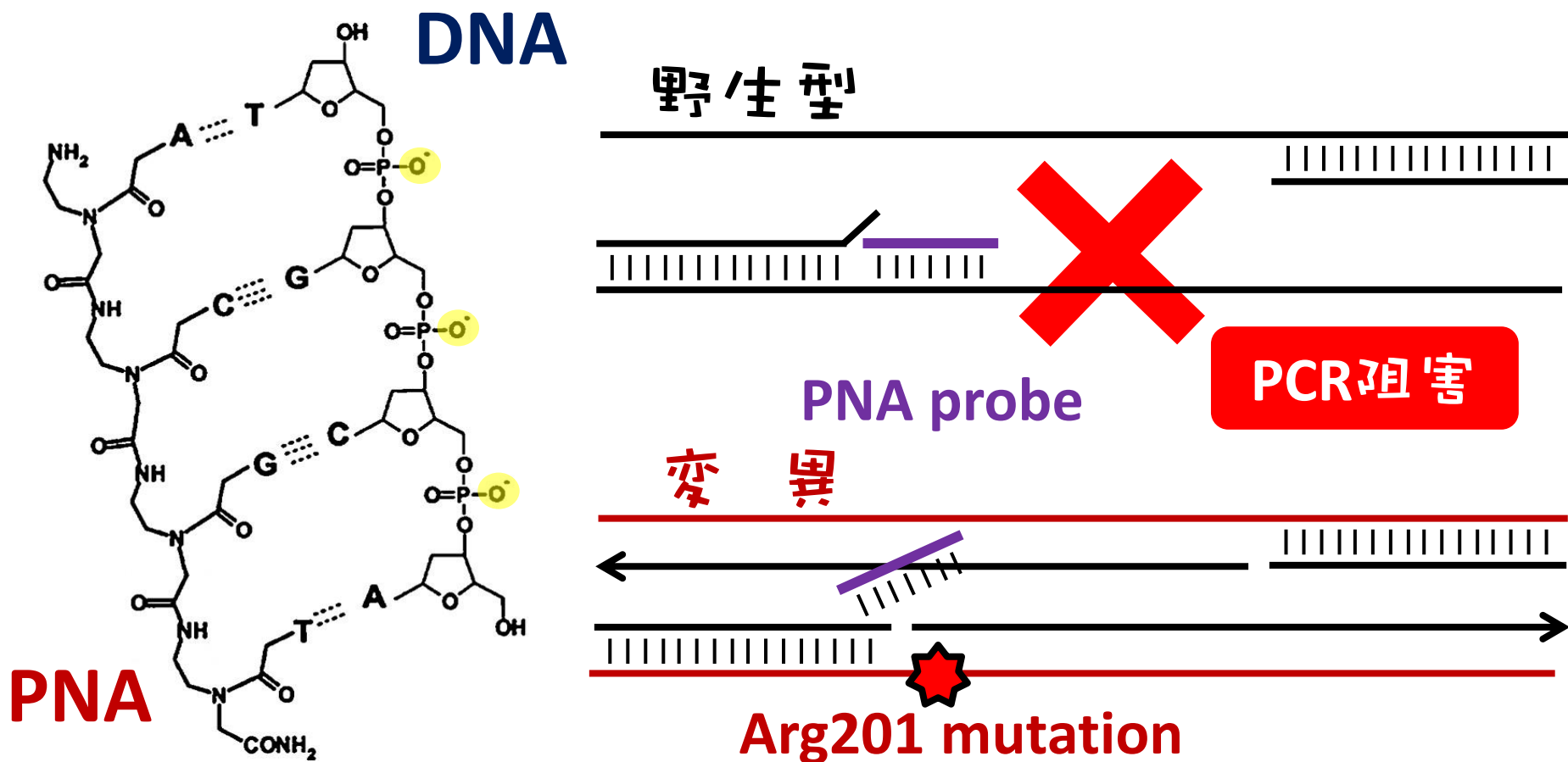
罹患組織（腫瘍）

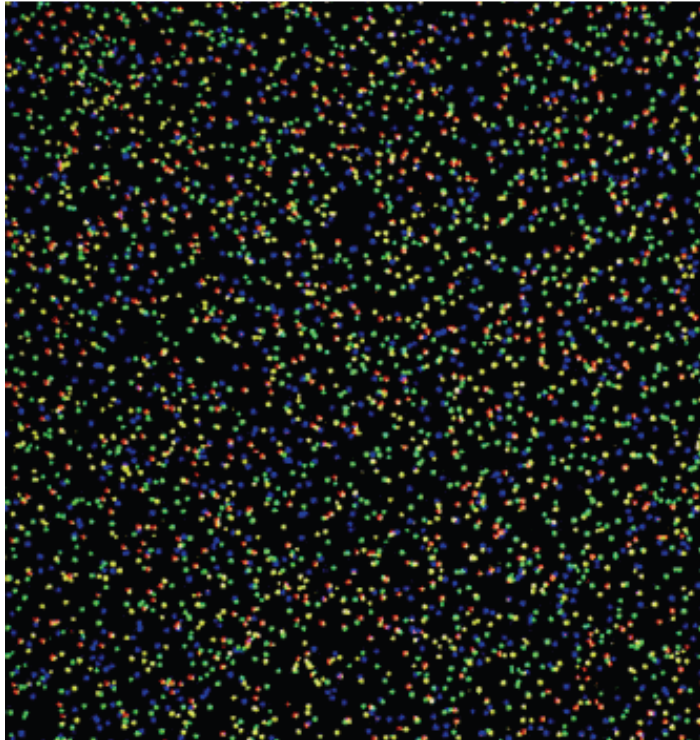
非罹患組織（末梢血など）



従来型遺伝子診断 (末梢血)

ペプチド核酸(PNA)法などによる変異濃縮

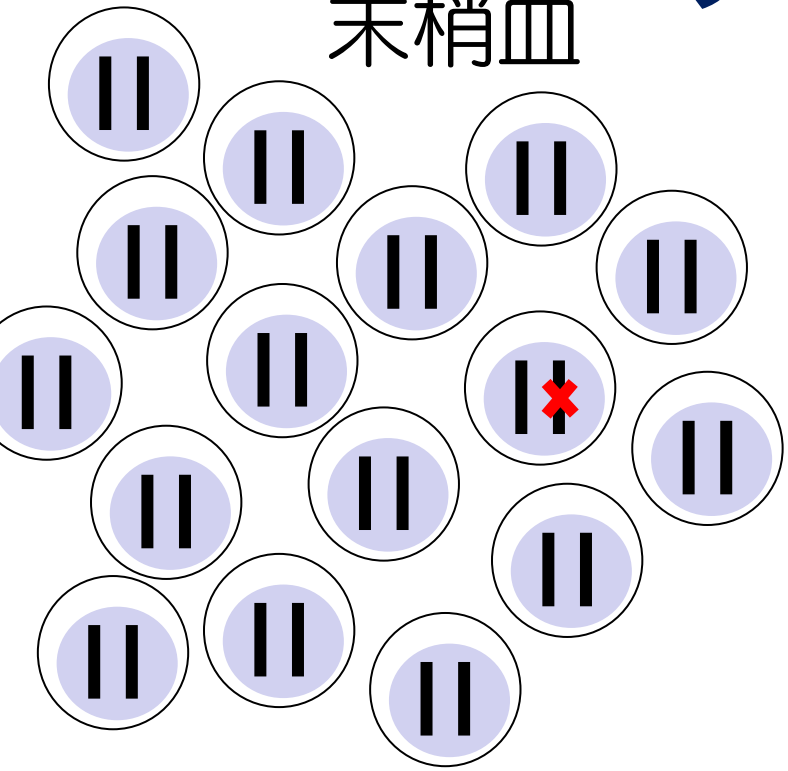




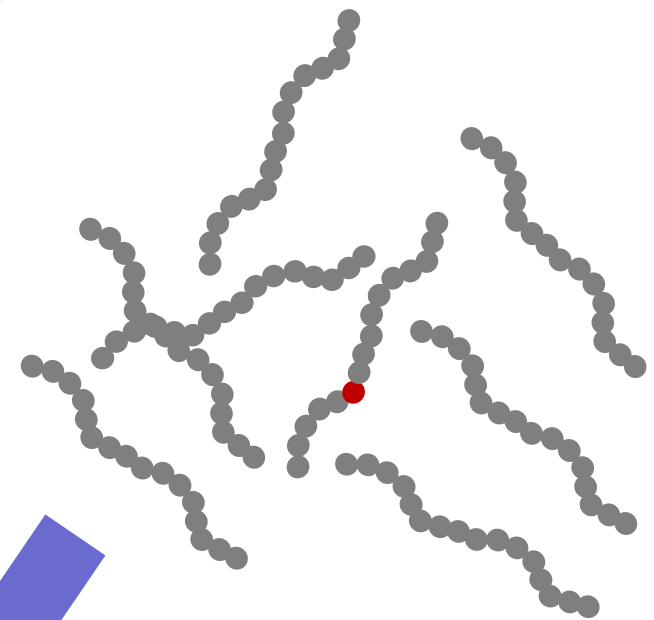
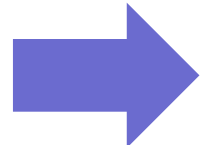
**Massively
Parallel
Sequencing**

アイディア

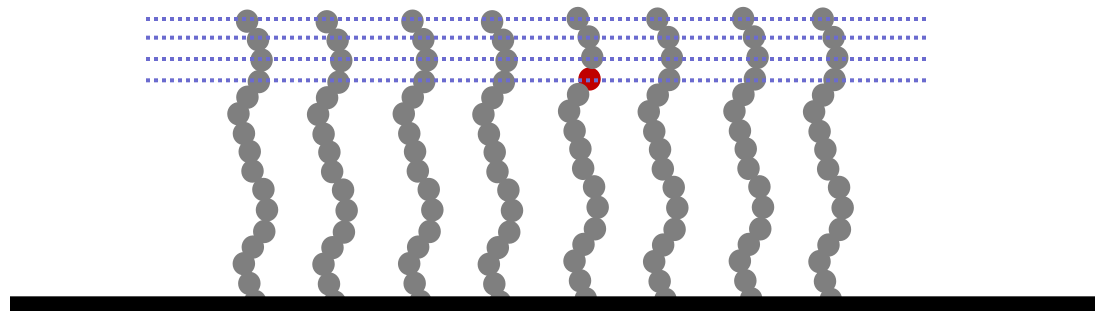
末梢血



PNA-PCR



NGS
(SBS)



Menu

イントロダクション

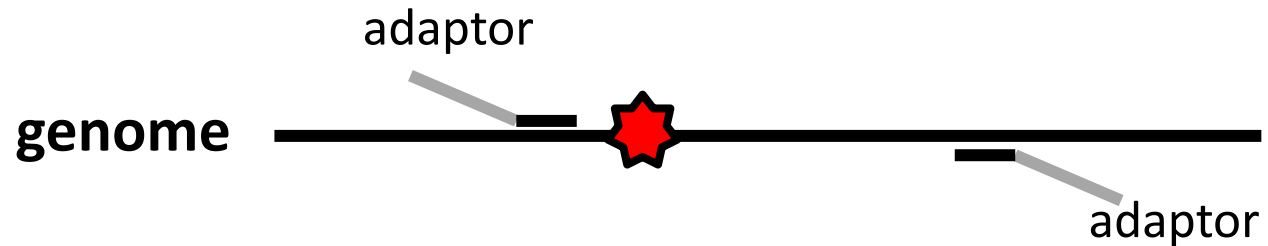
疾患について

ワークフロー

今後の展望



1st PCR



Forward primer:

CTACACGACGCTCTTCCGATCT**GTTTCA**
GGACCTGCTTCGC

Reverse primer:

GTGACTGGAGTTCAGACGTGTGCTCTTC
CGATCT**TCCACTTGCGGCGTTCATC**

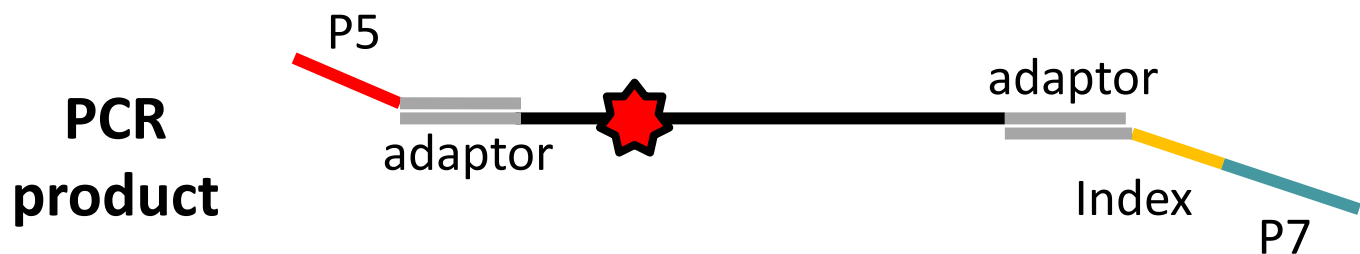
	Vol (uL)
Water	3.7
5x Buffer	2
100 mM dNTP	0.1
Herculase II (Agilent)	0.2
Primer FR mix (20 uM)	0.5
PNA probe (100 uM)	3
DNA (50 ng/uL)	0.5

Total 10 uL

98C 60 s → $\left. \begin{array}{l} 98C 10 s \\ 68C 60 s \\ 55C 30 s \\ 72C 30 s \end{array} \right\} \times 35 \rightarrow 72C 5 min$



2nd PCR



Forward primer:

AATGATACGGCGACCACCGAGATCTACAC
 TCTTTCCCTACACGACGCTCTTCCGATCT

Reverse primer:

CAAGCAGAAGACGGCATAACGAGAT
NNNNNNGTGACTGGAGTTCAGACGTGT
 Index

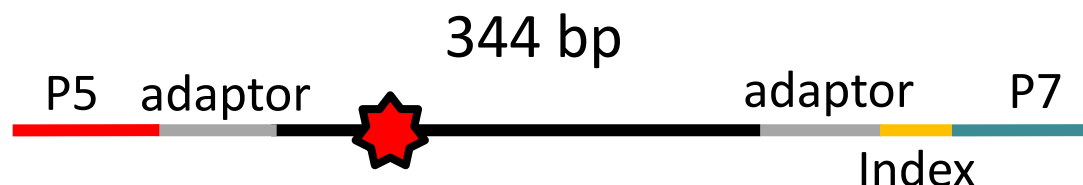
	Vol (uL)
Water	6.2
5x Buffer	2
100 mM dNTP	0.1
Herculase II (Agilent)	0.2
Primer FR mix (20 uM)	0.5
Diluted PCR product	1

Total 10 uL

98C 60 s → $\left. \begin{array}{l} 98C 10 s \\ 55C 30 s \\ 72C 30 s \end{array} \right\} \times 15 \rightarrow 72C 5 min$

Ampure精製

NGS

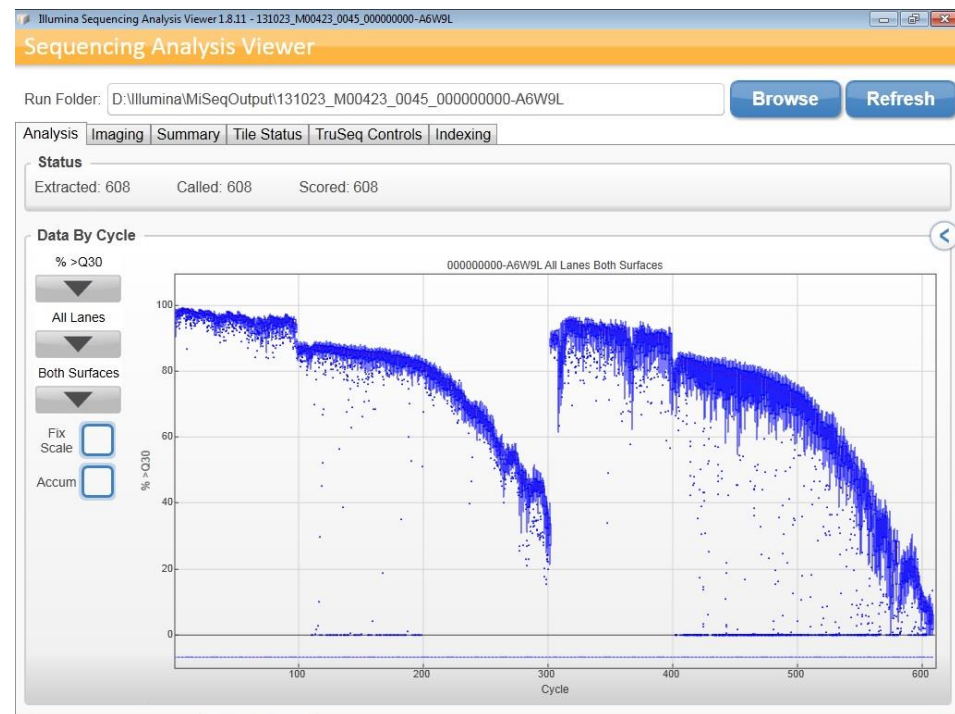
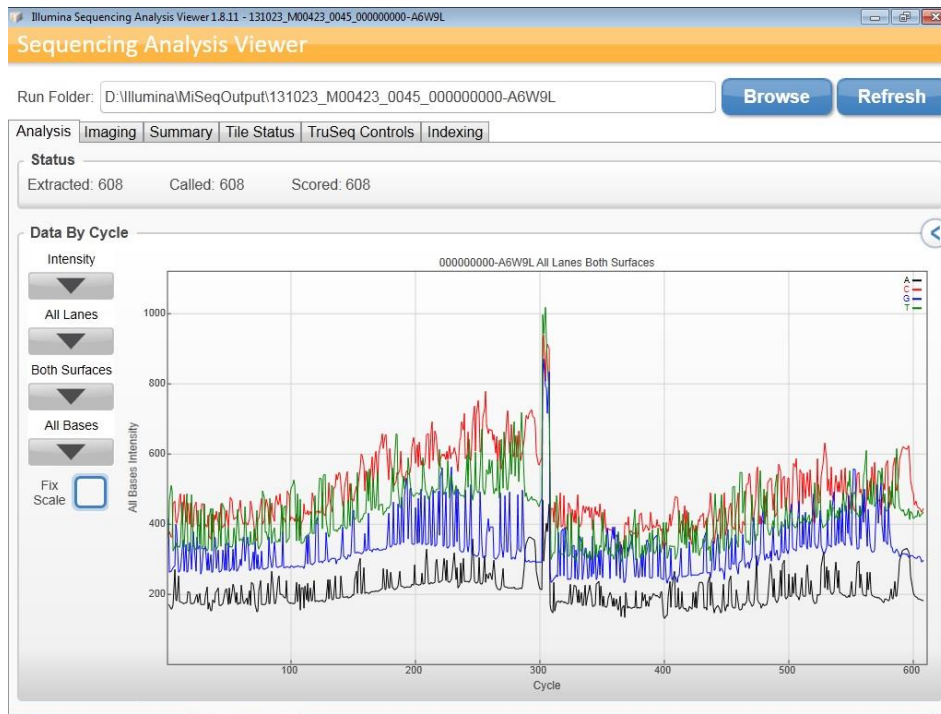


- * Indexの利用によりマルチプレックス解析可能
- * 安定したベースコールを得るため「多様性のあるライブラリ」（PhiXコントロールなど）を1/3以上スパイクインする
- * 50塩基以上のペアエンド解析で、2か所のホットスポット（Arg201, Gln227）をカバー

MiSeq V3 Cartridge (300 bp PE)

Intensity

Q30



Data analysis

マシン Xeon 2.5 GHz (24 cores); 128G RAM

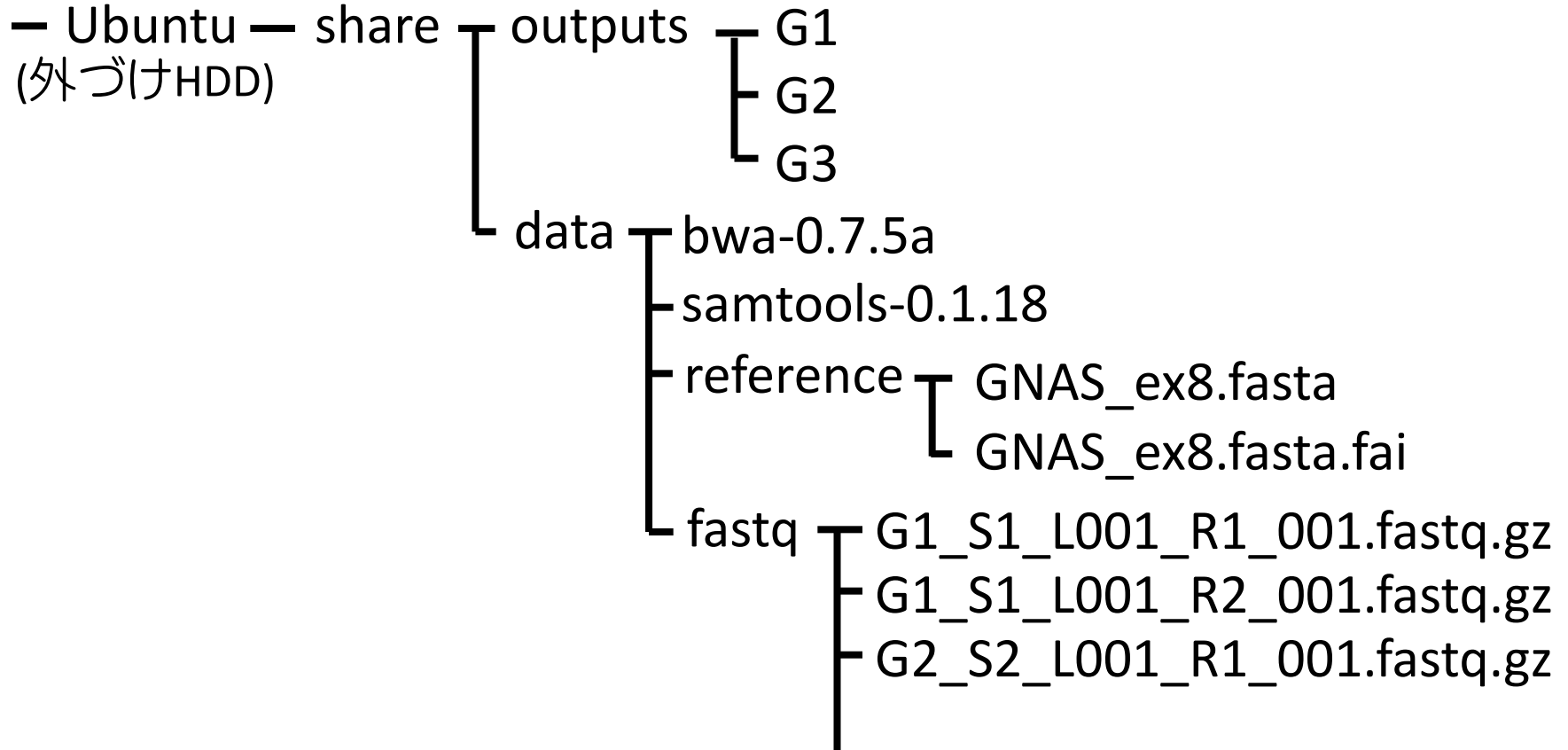
OS (dual boot)

Windows 7 Professional (64 bit)

Ubuntu 12.04 LTS (64 bit)

外づけHDD (2TB) Linux専用

Data analysis



Data analysis

準備 (変数の定義と出力格納ディレクトリの作成)

2,3,4...適宜変更可

```
i1=1  
out=/media/Ubuntu/share/outputs/  
fastq=/media/Ubuntu/share/data/fastq/  
reference=/media/Ubuntu/share/data/reference/GNAS_ex8.fast  
a  
  
mkdir /media/Ubuntu/share/outputs/G"$i1"/
```

Data analysis

BWA0.7によるマッピング

```
cd $out/G"$i1"  
bwa mem $reference $fastq/G"$i1"_S"$i1"_L001_R1_001.fastq.  
gz $fastq/G"$i1"_S"$i1"_L001_R2_001.fastq.gz -t 24 -M -R  
"@RG\tID:01\tSM:"$i1"\tPL:Illumina" > G"$i1".sam
```

→ ペアエンドマップ済みファイル (G1.sam)
が作成される

Data analysis

SAMtoolsによるbam変換、ソート、
インデックス作成（**mpileup**の準備）

```
cd $out/G"$i1"  
samtools view -bS G"$i1".sam > G"$i1".bam  
samtools sort G"$i1".bam G"$i1"_sorted  
samtools index G"$i1"_sorted.bam
```

→ ソート済みbamファイル (G1_sorted.bam)
とインデックスファイルが作成される

Data analysis

-BQオプション (BQのカットオフ値)を35に設定

-dオプション (最大depth)を大きな値に設定
(デフォルトは8000)

カウント

```
samtools mpileup -BQ 35 -uf $reference/GNAS_ex8.fasta  
-d 1000000 G"$i1"_sorted.bam |bcftools view -cg - >  
G"$i1".vcf
```

.vcf形式での出力を指定

→ 最終的な出力ファイル G1.vcf が作成される

Data analysis

The screenshot shows a Microsoft Excel spreadsheet with the following data columns: A (Sample ID), B (Read Count), C (Base), D (Quality), E (Position), F (DP), G (VDB), H (AF), I (AC), J (MQ), K (FQ), L (PV), M (P). The data rows range from 269 to 296. A red box highlights the 'DP4' values in column H, and a red callout bubble points to it with the text 'DP4に注目'.

	A	B	C	D	E	F	G	H	J	K	L	M
269	GNAS_ex8	266	A	.	28.2	.	DP=1 ;AC1=2;FQ=-30		PL	0		
270	GNAS_ex8	408	T	.	28.2	.	DP=45;VDB=0.0000;AC1=2;FQ=-30		PL	0		
271	GNAS_ex8	409	G	.	54	.	DP=481156;VDB=0.0000;AF1=0;AC1=	DP4=0,8,0,0;MQ=20;FQ=-51	PL	0		
272	GNAS_ex8	410	T	.	51	.	DP=483288;VDB=0.0000;AF1=0;AC1=	DP4=0,7,0,0;MQ=20;FQ=-48	PL	0		
273	GNAS_ex8	411	T	.	54	.	DP=483379;VDB=0.0000;AF1=0;AC1=	DP4=0,8,0,0;MQ=20;FQ=-51	PL	0		
274	GNAS_ex8	412	T	.	54	.	DP=483393;VDB=0.0000;AF1=0;AC1=	DP4=0,8,0,0;MQ=20;FQ=-51	PL	0		
275	GNAS_ex8	413	C	.	178	.	DP=483398;VDB=0.0000;AF1=0;AC1=	DP4=41,8,0,0;MQ=20;FQ=-175	PL	0		
276	GNAS_ex8	414	A	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=471450,6,114,0;MQ=20;FQ=-282;PV4=1,8.6e-08,1,0.46	PL	0		
277	GNAS_ex8	415	G	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=472714,6,128,0;MQ=20;FQ=-282;PV4=1,0.0036,1,1	PL	0		
278	GNAS_ex8	416	G	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=467747,7,138,0;MQ=20;FQ=-282;PV4=1,1.6e-05,1,0.054	PL	0		
279	GNAS_ex8	417	A	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=470835,5,86,0;MQ=20;FQ=-282;PV4=1,2.4e-12,1,1	PL	0		
280	GNAS_ex8	418	C	.	209	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=468700,7,90,0;MQ=20;FQ=-282;PV4=1,4.7e-39,1,1	PL	0		
281	GNAS_ex8	419	C	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=471782,7,68,0;MQ=20;FQ=-282;PV4=1,0.28,1,1	PL	0		
282	GNAS_ex8	420	T	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=465197,7,93,0;MQ=20;FQ=-282;PV4=1,4.8e-07,1,0.23	PL	0		
283	GNAS_ex8	421	G	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=448792,7,206,0;MQ=20;FQ=-282;PV4=1,0.0077,1,1	PL	0		
284	GNAS_ex8	422	C	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=468134,7,51,0;MQ=20;FQ=-282;PV4=1,2.2e-05,1,0.083	PL	0		
285	GNAS_ex8	423	T	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=452950,8,86,0;MQ=20;FQ=-282;PV4=1,0.1,1,1	PL	0		
286	GNAS_ex8	424	T	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=454729,8,45,0;MQ=20;FQ=-282;PV4=1,0.001,1,1	PL	0		
287	GNAS_ex8	425	C	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=451653,8,47,0;MQ=20;FQ=-282;PV4=1,0.22,1,0.075	PL	0		
288	GNAS_ex8	426	G	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=431964,8,382,0;MQ=20;FQ=-282;PV4=1,3.5e-05,1,0.019	PL	0		
289	GNAS_ex8	427	C	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=438976,8,22,0;MQ=20;FQ=-282;PV4=1,1,1,1	PL	0		
290	GNAS_ex8	428	T	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=463006,8,62,0;MQ=20;FQ=-282;PV4=1,8.9e-06,1,1	PL	0		
291	GNAS_ex8	429	G	.	202	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=457538,8,850,0;MQ=20;FQ=-282;PV4=1,7.2e-09,1,0.087	PL	0		
292	GNAS_ex8	430	C	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=446076,8,57,0;MQ=20;FQ=-282;PV4=1,1,1,1	PL	0		
293	GNAS_ex8	431	C	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=457107,8,82,0;MQ=20;FQ=-282;PV4=1,0.034,1,1	PL	0		
294	GNAS_ex8	432	G	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=440538,8,411,0;MQ=20;FQ=-282;PV4=1,0.064,1,1	PL	0		
295	GNAS_ex8	433	T	.	205	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=445639,8,58,0;MQ=20;FQ=-282;PV4=1,3.5e-09,1,1	PL	0		
296	GNAS_ex8	434	G	.	209	.	DP=483400;VDB=0.0000;AF1=0;AC1=	DP4=429081,8,77,0;MQ=20;FQ=-282;PV4=1,0.022,1,0.18	PL	0		

DP4に注目

Data analysis

DP=935526;VDB=0.0000;AF1=0;AC1=0 **DP4=435819,360988,376,302**;MQ=-2147483648;FG

DP4=435819, 360988, 376, 302

Forward
標準アリル数
Reverse
標準アリル数

Reverse
非標準アリル数
Forward
非標準アリル数

解析例

McCune-Albright症候群 疑い例 3

コントロール 3

	601 C	602 G	603 T
Cont 1	777757 / 150	781905 / 265	776532 / 101
Cont 2	804960 / 144	807788 / 256	802655 / 106
Cont 3	801748 / 112	806606 / 244	801504 / 98
Patient 1	826037 / 140	826973 / 724	823554 / 99
Patient 2	894561 / 151	897832 / 430	893605 / 149
Patient 3	1007221 / 249	1011798 / 716	1004996 / 145

Quantitative and Sensitive Detection of *GNAS* Mutations Causing McCune-Albright Syndrome with Next Generation Sequencing

Satoshi Narumi¹*, Kumihiko Matsuo², Tomohiro Ishii¹, Yusuke Tanahashi², Tomonobu Hasegawa¹

¹ Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan, ² Department of Pediatrics, Asahikawa Medical University, Hokkaido, Japan

Abstract

Somatic activating *GNAS* mutations cause McCune-Albright syndrome (MAS). Owing to low mutation abundance, mutant-specific enrichment procedures, such as the peptide nucleic acid (PNA) method, are required to detect mutations in peripheral blood. Next generation sequencing (NGS) can analyze millions of PCR amplicons independently, thus it is expected to detect low-abundance *GNAS* mutations quantitatively. In the present study, we aimed to develop an NGS-based method to detect low-abundance somatic *GNAS* mutations. PCR amplicons encompassing exons 8 and 9 of *GNAS*, in which most activating mutations occur, were sequenced on the MiSeq instrument. As expected, our NGS-based method could sequence the *GNAS* locus with very high read depth (approximately 100,000) and low error rate. A serial dilution study with use of cloned mutant and wildtype DNA samples showed a linear correlation between dilution and measured mutation abundance, indicating the reliability of quantification of the mutation. Using the serially diluted samples, the detection limits of three mutation detection methods (the PNA method, NGS, and combinatory use of PNA and NGS [PNA-NGS]) were

PLoS One. 2013;8(3):e60525. doi: 10.1371/journal.pone.0060525

変異検出限界 (変異アリル頻度)

PNA法

1%

NGS単独

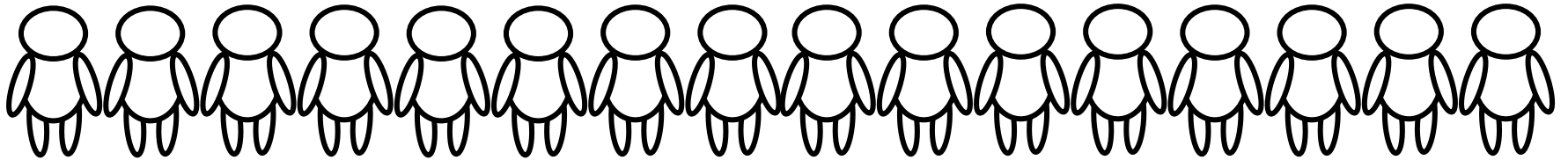
0.03%

PNA-NGS

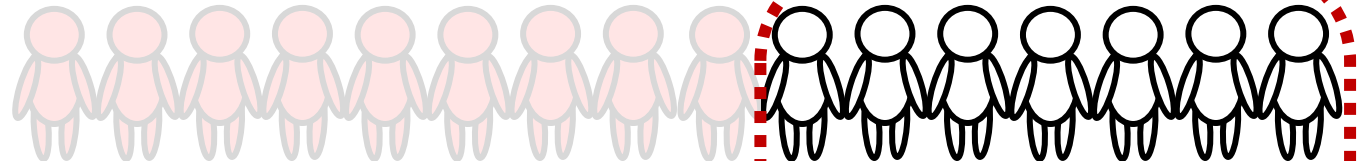
0.01%

変異検出感度が **100倍** アップ

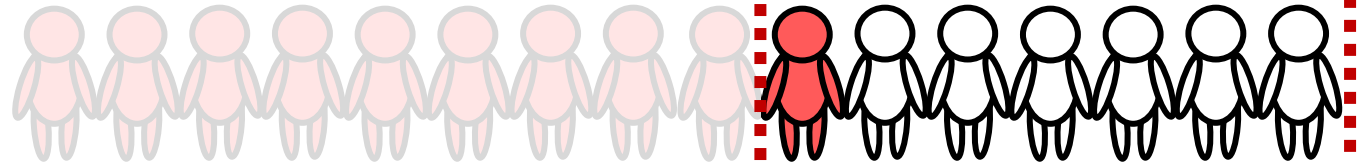
McCune-Albright症候群 患者末梢血DNA 16検体を遺伝子診断



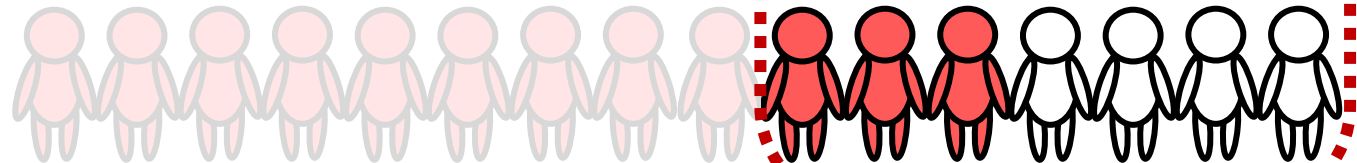
PNA法



NGS单独

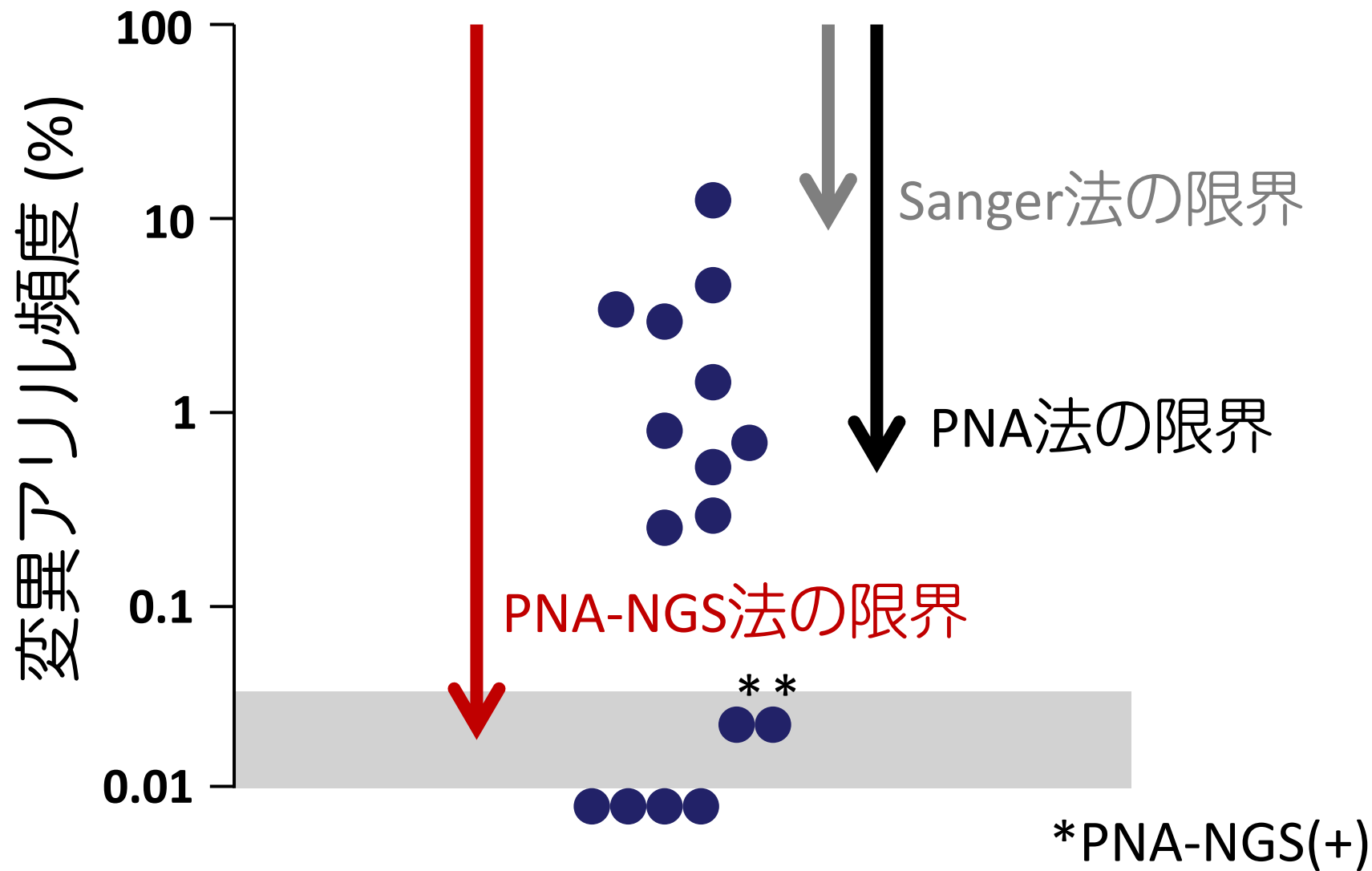


PNA-NGS



従来法陰性**7例中3例**で変異を検出

変異アリル頻度の分布



Menu

イントロダクション

疾患について

ワークフロー

今後の展望



デジタルPCRとの比較

- * ドロップレット内部で1コピーのDNAを鋳型にPCR (Taqmanプローブ)
 - * NGSに次ぐ、もうひとつの「デジタル」
 - * 低頻度変異の検出限界 **0.001%***
 - *出典 BioRad社ウェブサイト (BRAF V600Eでの検討)
<http://www.bio-rad.com/ja-jp/product/qx100-droplet-digital-pcr-system>
- 変異部位を事前に予測できることが必要
- * 現時点では高コスト・低スループット(?)

体細胞変異を病因とする先天異常

Proteus症候群 (*AKT1*)

ORIGINAL ARTICLE

A Mosaic Activating Mutation in *AKT1*
Associated with the Proteus Syndrome

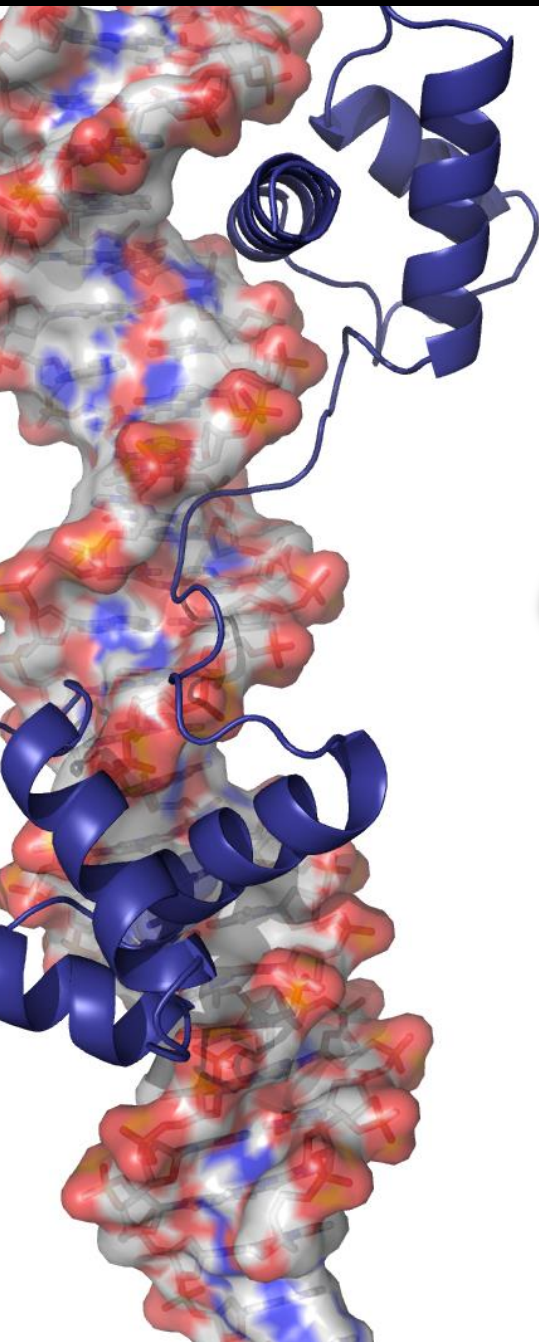


Sturge-Weber症候群 (*GNAQ*)

ORIGINAL ARTICLE

Sturge-Weber Syndrome and Port-Wine Stains
Caused by Somatic Mutation in *GNAQ*





Thanks