

## スライド1

### ○ スライド一覧

☰ Lysine residues in human adenylate kinase are essential for interaction with adenine nucleotides as found by site-directed random mutagenesis

⇒ Takanori Ayabe

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⇒ Department of Hygiene, The Second Department of Surgery, Miyazaki Medical College

⇒ Porcine Adenylate Kinase

⇒ ADENYLATE KINASE ISOENZYME 1 (EC 2.7.4.3) (ATP-AMP TRANSPHOSPHORYLASE)

⇒ DE (AK1) (MYOKINASE).

⇒ OS SUS SCROFA (PIG).

⇒ DR PDB; 3ADK; 16-APR-88.

⇒ [ENTRY / RASMOL / 3D IMAGE / HSSP ENTRY / SCOP]

⇒ DR SWISS-PROT; P00571; KAD1\_PIG.

⇒ KW TRANSFERASE; KINASE; ATP-BINDING; ACETYLATION; 3D-STRUCTURE.

☰ ヒトアデニル酸キナーゼ(AK1)

⇒

⇒ 1) 194アミノ酸から構成されるリン酸転移酵素である。

⇒ 2) 分子量21,700の球状蛋白質である。

⇒ 3) 2つのアデニンヌクレオチド基質結合サイトをもつ。  
(MgATPサイトとAMPサイト)

⇒ 4) 3つのアイソザイムが存在する(AK1, Ak2, AK3)。

AK1は細胞質に存在し、骨格筋、脳、赤血球にみられる。

AK2は、ミトコン

ドリア膜間スペースに、AK3は、ミトコンドリ

⇒ アマトリックスに存在し、肝臓、腎臓にみられる。

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- 5)赤血球内AK1欠損症による溶血性貧血を引き起こす遺伝性疾患 (Miwa et al., 1983)や、また、AK1の点変異による溶血性貧血 (Arg128からTrpへの置換)(Matsuura et al., 1989)などが報告されている。

### ☰ Established sources of members of the adenylate kinase family

Species	Source	Characterization	Established	Sequence	Reference
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Yeast	cytosol	1967	1986		Tomasselli et al.
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Escherichia coli		1973	1985		Brune et al.

### ☰ アデニル酸キナーゼの構造と機能解析の歴史的背景

- 1. X-ray crystallographic study (Schulz et al., 1974, Pai et al., 1977, Egner et al, 1987, Schulz et al., 1990 )

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- ⇒ 2. NMR study  
(Mildvan & Fry et al., 1987, Yan & Tsai, 1991)
- ⇒ 3. Kinetic analysis of peptide fragment (Kuby et al., 1978)
- ⇒ 4. Site-directed mutagenesis  
(Kim et al., 1990, Yoneya et al., 1990, Yan & Tsai, 1991)
- ☰ AK1のアミノ酸一次構造の比較
  - ⇒ Human (von Zabern et al., 1976)
  - ⇒ Rabbit (Kuby et al., 1984)
  - ⇒ Calf (Kuby et al., 1984)
  - ⇒ Porcine (Heil et al., 1974)
  - ⇒ Chicken (Kishi et al., 1986)
- ☰ 本研究の目的
  - ⇒ 1)ヒト骨格筋AKとATPの相互作用を調べるために、哺乳類動物間で保存性の高いリジン残基7つ(K9, K21, K27, K31, K63, K131, K194)を選び、ヒトアデニル酸キナーゼ人工合成遺伝子を用いて、部位特異的変異導入を行う。  
⇒
  - ⇒ 2)アミノ酸の置換の方法は、標的リジン残基に対し、プライマーのデザインを工夫し、変異体をランダムに複数作製し、複数の変異体を短時間で蛋白質発現、及び、精製する系を確立する。  
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  - ⇒ 3)野生型酵素と変異型酵素の性質を、酵素反応速度論的に解析し、AK構造モデルにおいて、リジン残基を置換し得た変異型と野生型酵素の基質との相互作用を考察する試みを行う。
- ☰ DNA sequence of hAK1 gene  
(Kim et al., 1989)
- ☰ Construction of pMEX8-hAK1
- ☰ DNA sequence of primer  
□

## スライド1

- ⇒ Lys9            5'-CGAAGATGATYXXAGTCTTCTTAAGC-3'
- Lys21            5'-GCACTGGGTACCYXXGCCAGAACC-3'
- Lys27            5'-GCACGATYXXCTCGCACTGGG -3'
- Lys31            5'-GTGTAGCCGTAYXXCTGCACGATTTTC-3'
- Lys63            5'-CCAGCTGACCYXXTTCCATGATTTTC-3'
- Lys131           5'-GTTTCGCCGCGYXXCAGCAGGCG-3'
- Lys194           5'-CGAAGATGATYXXAGTCTTCTTAAGC-3'

⇒

X: A, G, C, T      Y: G, C

### ☰ Site-directed mutagenesis

#### ☰ Screening of mutant pMEX8-hAK1

- ⇒ 1. Transformation with homoduplex DNA and TG1 competent cells
- ⇒ 2. Culture on LB-plate with ampicillin (50µg/ml)
- ⇒ 3. Small culture of LB medium (overnight)
- ⇒ 4. Plasmid purification (Sephaglass, alkaline method)
- ⇒ 5. DNA Cycle Sequence by PCR

⇒

⇒

#### Reaction mixture

- ⇒ AmpliTaq DNA Polymerase : Taq DNA Polymerase = 9:1 (2U/2µl)
- ⇒ double-stranded template DNA (2.8 µg)
- ⇒ Fluorescent isothiocyanate-labeled sequence primer (2 pmol)
- ⇒ Termination Mixes (ddATP, ddGTP, ddCTP, ddTTP)

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#### PCR Condition

- ⇒ 1 cycle 95 for 5 sec

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- ▢ 20 cycle 95 for 30 sec
- ▢ 53 for 30 sec
- ▢ 72 for 60 sec
- ▢ 20 cycle 95 for 30 sec
- ▢ 72 for 60 sec
- ☰ Results: DNA sequence of Lys9-mutants
- ☰ Results: DNA sequence of Lys21- and Lys63- mutants
- ☰ Results: DNA sequence of Lys27-mutants
- ☰ Results: DNA sequence of Lys31-mutants
- ☰ Results: DNA sequence of Lys131-mutants
- ☰ Results: DNA sequence of Lys194-mutants
- ☰ Results of site-directed mutagenesis
- ☰ Protein expression and purification of wild-type and mutant AKs
  - ▢ 1. Transformation with plasmid DNA and TG1 cells
  - ▢ 2. Small culture of the cells in LB medium (5 ml ) overnight
  - ▢ 3. Culture of the medium in 250 ml for 1 hr
  - ▢ 4. Addition of isopropyl-  $\beta$ -D-thio-galactopyranoside (IPTG)  
(a final concentration of 1 mM)
  - ▢ 5. Culture the medium for 16 hr
  - ▢ 6. Centrifugation of the medium (5,000 X g for 20 min)
  - ▢ 7. Disruption of the pellet of E.coli cells in standard buffer  
(10ml) (Ultrasonicator, 20kHz, 20W, 3min)
  - ▢ 8. Centrifugation of the homogenate (12,000 X g for 20 min)
  - ▢ 9. Blue sepharose CL-6B column chromatography
  - ▢ 10. 12.5% SDS-PAGE and concentration
  - ▢ 11. Gel filtration (Superose 12)
  - ▢ 12. 12.5% SDS-PAGE
  - ▢ 13. Measurement of the concentration of protein (Lowry method)
  - ▢ 14. Kinetic analysis of forward reaction of AK

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### ☰ Blue Sepharose Chromatography

- ☐ Column: Blue Sepharose CL-6B ( 1 X 5 cm)
- ☐ Standard buffer: 20mM Tris-HCl, 1 mM EDTA,  
☐ 0.1 mM dithiothreitol, pH 7.4
- ☐ Gradient: 0 - 1 M NaCl
- ☐ Velocity: 0.5 ml/min
- ☐ Fraction size: 3 ml

### ☰ Superose 12 Column Chromatography

- ☐ Column: Superose 12 ( 1 X 30 cm )
- ☐ Imidazole buffer: 5mM imidazol-HCl, 1 mM EDTA,  
☐ 0.1 mM dithiothreitol, pH 6.9
- ☐ Velocity: 0.5 ml/min
- ☐ Fraction size: 2 ml
- ☐

### ☰ Results of protein purification

#### ☰ AK assay

- ☐
- ☐ Forward Reaction
- ☐ <Reaction Mixture (1ml) of the ADP formation reaction>
  - ☐ 75mM Triethanolamine HCl (pH 7.5)
  - ☐ 120mM KCl, 0.2mM NADH
  - ☐ 0.3mM Phosphoenolpyruvate, 2mM MgSO<sub>4</sub>
  - ☐ 2mM ATP, (2, 1, 0.5, 0.33, 0.25) mM AMP
  - ☐ 0.3 mg/ml Bovine serum albumine
  - ☐ 15U Lactate dehydrogenase, 6U Pyruvate kinase
  - ☐ AK sample

- ☐
- ☐
- ☐

AK, Mg<sup>2+</sup>

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- ▢ ATP + AMP → 2 ADP
- ▢
- ▢
- ▢ Pyruvate Kinase (PK)
- ▢ 2 ADP + 2 Phosphoenolpyruvate (PEP) → 2 ATP + 2 Pyruvate
- ▢
- ▢
- ▢ Lactate Dehydrogenase (LDH)
- ▢ 2 Pyruvate + 2 NADH + 2H<sup>+</sup> → 2 L-Lactate + 2 NAD<sup>+</sup>
- ▢
- ▢ observe reduction of NADH at 340 nm

☰ double reciprocal plot

☰ Kinetic results of Lys-mutants

	kcat/Km MgATP	kcat/Km AMP	Km MgATP	Km AMP	kcat
▢ Lys9	-	-	-	-	- -
▢	- - -	- - - -			- - - -
▢	- - - -	- - - - -		+	- - - -
▢	- - - -	- - - -	++	++	- -
▢					

スライド1

▢	Lys21	K21P	+++	++	--
	- - -	- - -			
▢					
▢	Lys27	K27R			--
	- - -	- -			
▢		K27L	+		--
	- - -	- -			
▢		K27I	+		- - -
	- - - -	- - -			
▢					
▢	Lys31	K31F	++		--
	- - - -	- - -			
▢		K31I	-		--
	- -	- -			
▢		K31S	+	++++	- - -
	- - -	- - - -			
▢					
▢	Lys63	K63F	+	-	- - -
	- - - -	- -			
▢					
▢	Lys131	K131A	+++		- - -
	- - - -	- - -			
▢		K131F			- - -
	- - -	- - -			
▢					
▢	Lys194	K194S	++		-
	- -	-			
▢		K194I	-	-	--
	-	- -			



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□	K194L	+	-	-	
□	- -				
□	K194P		-	- - -	-
□	- -				
□	K194N			- -	
□	- -				
□	K194V	-	+	+	+
□	- -				
□	- - - -				
□					
□					
□	Kmの減少	Km < 1.0	-	kcat	10 % - 100 %
□	-				
□	Kmの増加	1.0 < Km < 5.0	空欄		1 % - 10
□	%	- -			
□		5.0 < Km < 10.0	+		0.10 % - 1
□	%	- - -			
□		10.0 < Km < 15.0	+		<
□	0.10 %	- - - -			
□		15.0 < Km < 20.0	+		
□		20.0 < Km < 25.0	+		

☰ まとめ

- 
- (1) ヒトAKをコードするcDNAを用い、比較的容易に短時間に複数の変異体を得る大腸菌発現系を確立した。
- 
- (2) リジン残基(K9, K21, K27, K31, K63, K131, K194)に対して、ランダムに部位特異的変異導入を行い、26種類の変異型酵素を得た。
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- (3)変異型酵素の酵素キネテックス解析により、基質(ATP, AMP)親和性及び触媒作用の増加や減少が観察され、リジン残基は、酵素活性に必須なアミノ酸残基であることが示唆された。

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- Porcine Adenylate Kinase
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- DE (AK1) (MYOKINASE).
- OS SUS SCROFA (PIG).
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- [ENTRY / RASMOL / 3D IMAGE / HSSP ENTRY / SCOP]
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# ヒトアデニル酸キナーゼ(AK1)



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- 2) 分子量21,700の球状蛋白質である。
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- 1. X-ray crystallographic study  
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- 4. Site-directed mutagenesis  
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# 本研究の目的

- 1) ヒト骨格筋AKとATPの相互作用を調べるために、哺乳類動物間で保存性の高いリジン残基7つ(K9, K21, K27, K31, K63, K131, K194)を選び、ヒトアデニル酸キナーゼ人工合成遺伝子を用いて、部位特異的変異導入を行う。
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# DNA sequence of hAK1 gene (Kim et al., 1989)

Lys9

Lys21

Lys27

Lys31

Lys63

Lys131

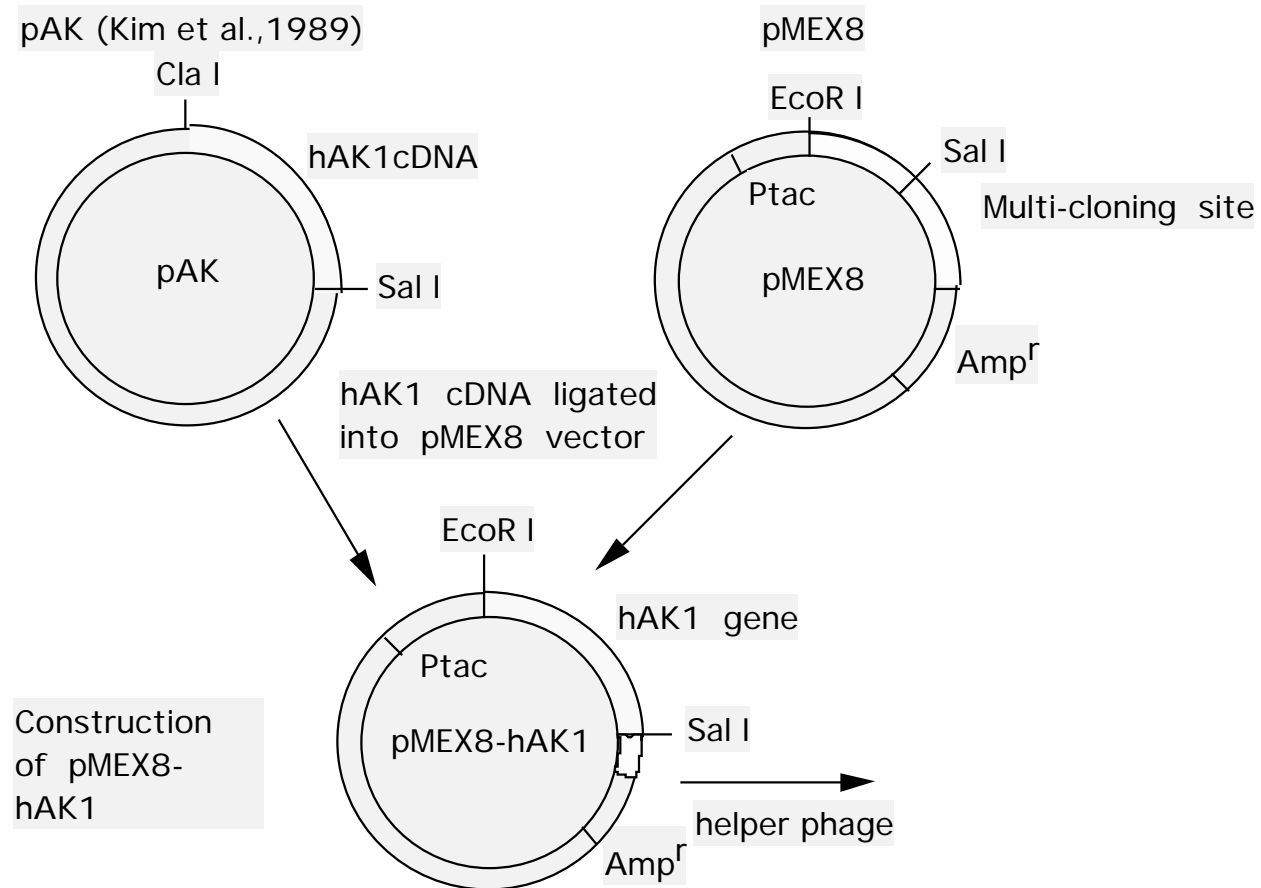
Lys194

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1      10      20      30      40      50      60      70      80      90      100
Met Glu Glu Lys Leu Lys Lys Thr Lys Ile Ile Phe Val Val Gly Gly Pro Gly Ser Gly
Cis 1  U1      A1111      U7      U7      U7      U7      U7      U7      U7      U7
cga1  ATG GAA GAG AAG GGT GAG AAG ACT AAG ATC ATC TTC GTT GTT GGC GGC GGC GGC TCT GGC
18  TAC CTT CTC TTC GAA TTC TTC TGA TTC TAG TAG AAG GAA GAA GGC CTT GGC GGA AGA GGC
      L1
Lys Gly Thr Gln Cys Glu Lys Ile Val Gln Lys Tyr Gly Tyr Thr His Leu Ser Thr Gly
      Kpn1 U3      U4      U4      U4      U4      U4      U4      U4      U4      U4
AAA GGT ATG CAG TGC GAG AAA ATC GTG CAG AAA TAC GGC TAC ACT CAC CTT TCT ACT GGT
      L2      L3      L4
TTT GAA TGC GTC ACC CTC TTT TAG CAC GTC TTT ATG GGC ATG TGA GTG GAC AGA TGA GCA
      L3      L4
Asp Leu Leu Arg Ser Glu Val Ser Ser Gly Ser Ala Arg Gly Lys Lys Leu Ser Glu Ile
      U5      Sac1/Sal1      U6      U6      U6      U6      U6      U6      U6      U6
GAC CTC CTC GGT TGC GAA GTC ACC TTT GGC TCT CCT GGT GGC AAG AAA CTC TCT GAA ATC
      L5      L5      L5      L5      L5      L5      L5      L5      L5      L5
CTG GAC GAC GCA ACC CTT CAC TCT ACC GGC AGA GGA GCA GGC TTC TTT GAC AGA CTT TAG
      L5
Met Glu Lys Gly Gln Leu Val Pro Leu Gln Thr Val Leu Asp Met Leu Arg Asp Ala Met
      U7      Pvu11      U8      U8      U8      U8      U8      U8      U8      U8
ATG GAA AAA GGT GAG CTC GTT GGC CTG GAG ACT GTT CTG GAC ATG CTG GGT GAC GGT ATG
      L7      L7      L7      L7      L7      L7      L7      L7      L7      L7
TAC CTT TTT CCA GTC GAC CAA GGC GAC CTC TGA CAA GAC CTG TAC GAC GCA CTG GAG TAC
      L7
Val Ala Lys Val Asn Thr Ser Lys Gly Phe Leu Ile Asp Gly Tyr Pro Arg Glu Val Gln
      U9      U10      U10      U10      U10      U10      U10      U10      U10      U10
GTT GCA AAA GTA AAG ACT TCT AAA GGC TTC CTG ATC GAC GGT TAC GGC GGC GAA GTT GAA
      L9      L9      L9      L9      L9      L9      L9      L9      L9      L9
GAA GGT TTT CAT TTG TGA AGA TTT GGC AAG GAC TAG CTG GCA ATG GGC GGC CTT GAA GTT
      L9
Gln Gly Glu Glu Phe Glu Arg Arg Ile Gly Gln Pro Thr Leu Leu Leu Tyr Val Asp Ala
      U11      EcoR1      U12      U12      U12      U12      U12      U12      U12      U12
GAA GGT GAA GGA TTC GAG GGC GGT ATC GGT GAC GGC ACT CTG CTT CTC TAG GTT GAT GGT
      L11      L11      L11      L11      L11      L11      L11      L11      L11      L11
GTT GCA CTT TTT AAG CTC GGC GCA TAG GCA GTC GGC TGA GAC GAA GAG ATG GAA CTA GCT
      L11
Gly Pro Glu Thr Met Thr Arg Arg Leu Leu Lys Arg Gly Glu Thr Ser Gly Arg Val Asp
      U13      U14      U14      U14      U14      U14      U14      U14      U14      U14
GCG GTC GAG ACT ATG ACT GGT GGC CTG CTG AAG GCG GCG GAA ACT TCG GGT GGC GTC GAC
      L13      L13      L13      L13      L13      L13      L13      L13      L13      L13
GCG GGT CTC TGA TAC TGA GCA GCG GAC GAC TTC GCG GCG CTT TGA ACC CCT GCG GAT CTC
      L13
Asp Asn Glu Glu Thr Ile Lys Lys Arg Leu Glu Thr Tyr Tyr Lys Ala Thr Glu Pro Val
      U15      U16      U16      U16      U16      U16      U16      U16      U16      U16
GAC AAC GAA GAG ACC ATT AAG AAA GGT CTG GAA ACC TAC TAC AAA GCT ACT GAA GCG GTT
      L15      L15      L15      L15      L15      L15      L15      L15      L15      L15
CTG TTG CTT CTC TGG TAA TTC TTT GCA GAC CTT TGG ATG ATG TTT GCA CTT GGC GAA
      L15
Ile Ala Phe Tyr Glu Lys Arg Gly Ile Val Arg Lys Val Asn Ala Glu Gly Ser Val Asp
      U17      U18      U18      U18      U18      U18      U18      U18      U18      U18
ATC GCT TTC TAC GAG AAA GGT GGT ATC GTT GCG AAA GTT AAG GGT GAA GGT TCT GTT GAC
      L17      L17      L17      L17      L17      L17      L17      L17      L17      L17
TAG GCA AAG ATG CTC TTT GCA CCA TAG GAA GCG TTT GAA TTC GCA CTT GCA AGA CAA CTC
      L17
Gly Val Phe Ser Gln Val Cys Thr His Leu Asp Ala Leu Lys Stop/Stop
      U19      Bac11      U20      U20      U20      U20      U20      U20      U20      U20
GAA GTA TTC TCT CAG GTA TCC ACT CAC CTG TGC GGT CTG AAA TAA TAG
      L19      L19      L19      L19      L19      L19      L19      L19      L19      L19
CTT CAT AAG AGA GTC CAT ACC TGA GTC GAC CTT GCA GAC TTT ATT ATC spst
      L19

```

# Construction of pMEX8-hAK1



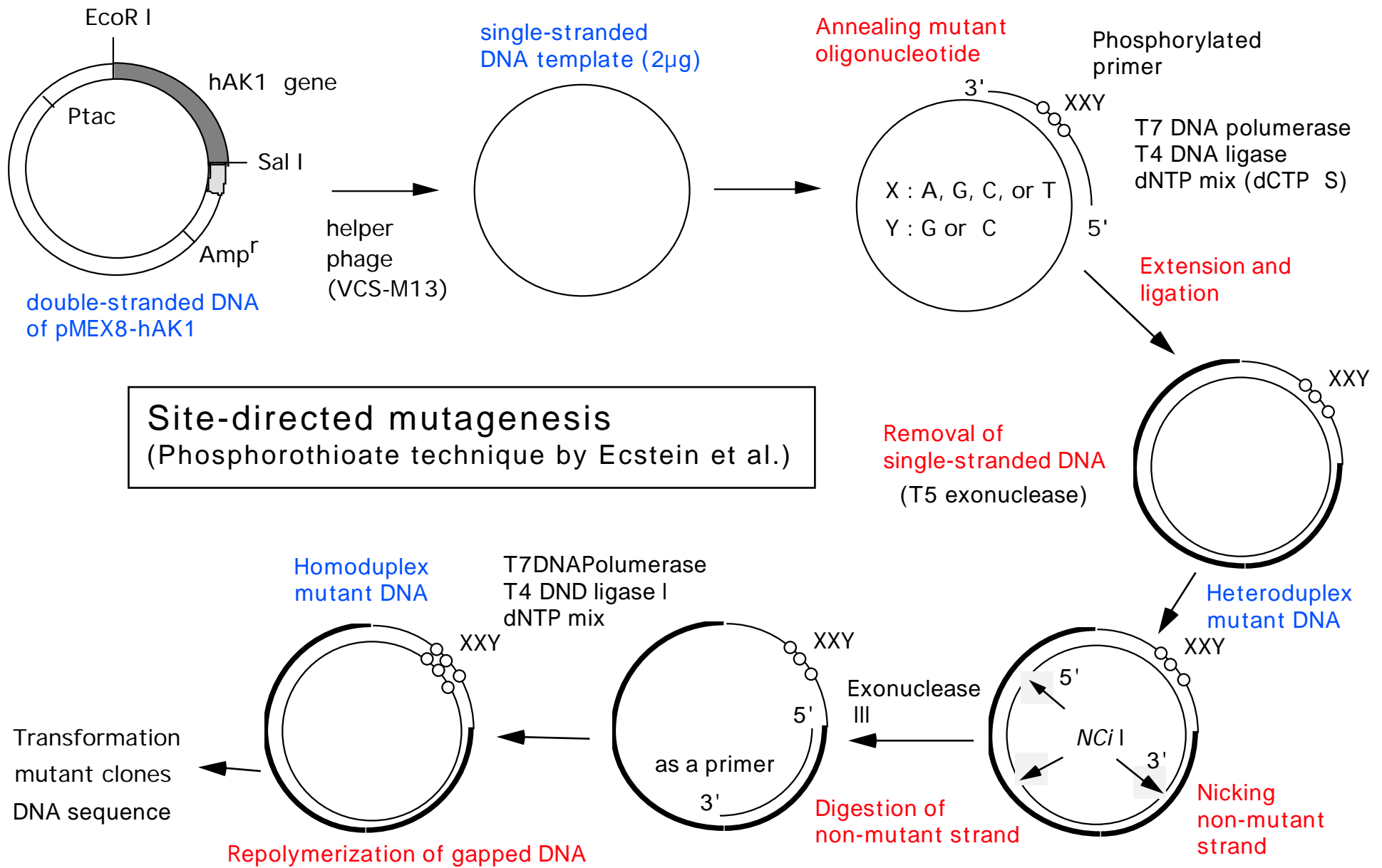
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  - Lys194** 5'-CGAAGATGATYXXAGTCTTCTTAAGC-3'
- 

X: A, G, C, T

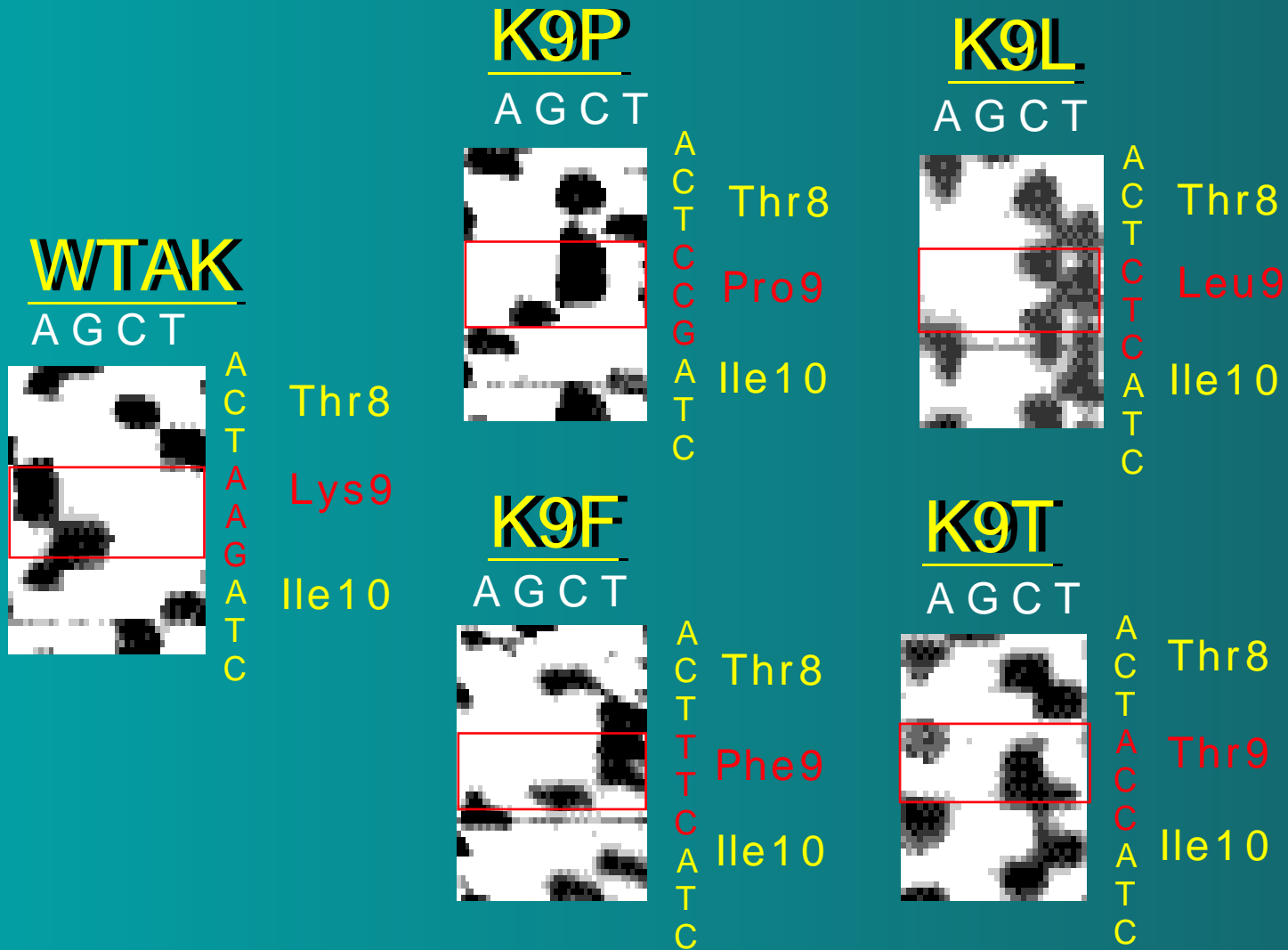
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- 4. Plasmid purification (Sephaglass, alkaline method)
- 5. DNA Cycle Sequence by PCR
- 
- 
- Reaction mixture
- • AmpliTaq DNA Polymerase : Taq DNA Polymerase = 9:1 (2U/2 $\mu$ l)
- • double-stranded template DNA (2.8  $\mu$ g)
- • Fluorescent isothiocyanate-labeled sequence primer (2 pmol)
- • Termination Mixes (ddATP, ddGTP, ddCTP, ddTTP)
- •                      PCR Condition
- •                      1 cycle    95    for 5 sec
- •                      20 cycle   95    for 30 sec
- •                                           53    for 30 sec
- •                                           72    for 60 sec
- •                      20 cycle   95    for 30 sec
- •                                           72    for 60 sec

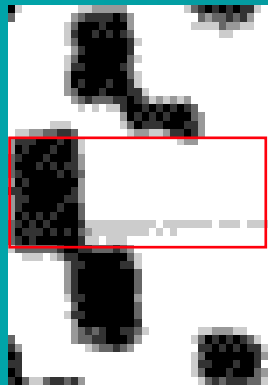
## Results: DNA sequence of Lys9-mutants



# Results: DNA sequence of Lys21- and Lys63- mutants

WTAK

A G C T



G  
G  
C  
A  
A  
A  
G  
G  
T

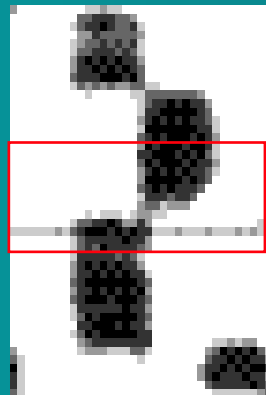
Gly20

Lys21

Gly22

K21P

A G C T



G  
G  
C  
C  
C  
G  
G  
G  
T

Gly20

Pro21

Gly22

WTAK

A G C T



G  
A  
A  
A  
A  
A  
G  
G  
T

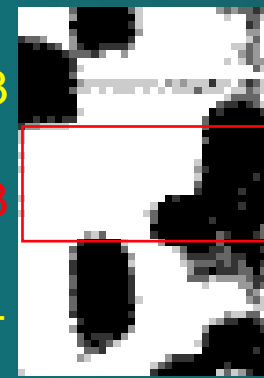
Glu63

Lys63

Gly64

K63F

A G C T



G  
A  
A  
T  
T  
C  
G  
G  
T

Glu62

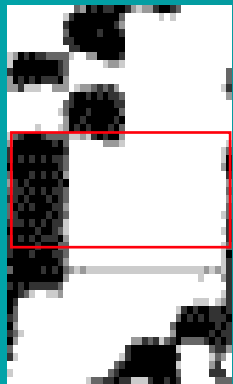
Phe63

Gly64

# Results: DNA sequence of Lys27-mutants

## WTAK

A G C T



G  
A  
G  
A  
A  
A  
A  
T  
C  
Glu26  
Lys27  
Ile28

## K27L

A G C T



G  
A  
G  
C  
T  
C  
A  
T  
C  
Glu26  
Lue27  
Ile28

## K27V

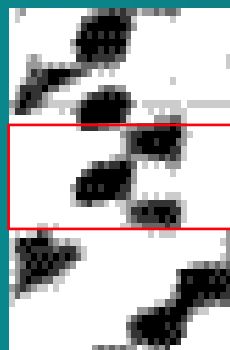
A G C T



G  
A  
G  
G  
T  
C  
A  
T  
C  
Glu26  
Val27  
Ile28

## K27R

A G C T



G  
A  
G  
C  
G  
C  
A  
T  
C  
Glu26  
Arg27  
Ile28

## K27I

A G C T



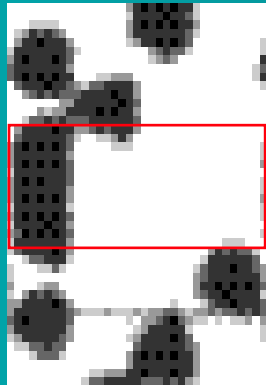
G  
A  
G  
A  
T  
A  
A  
T  
C  
Glu26  
Ile27  
Ile28



# Results: DNA sequence of Lys31-mutants

WTAK

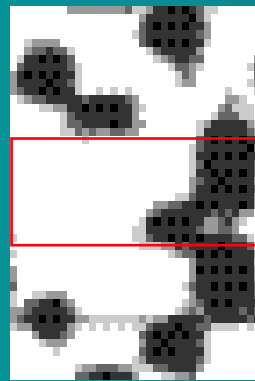
A G C T



C  
A Gln30  
G  
A Lys31  
A  
A Tyr32  
T  
A  
C

K31F

A G C T



C  
A Gln30  
G  
T Phe31  
T  
C  
T  
A  
C Tyr32

K31I

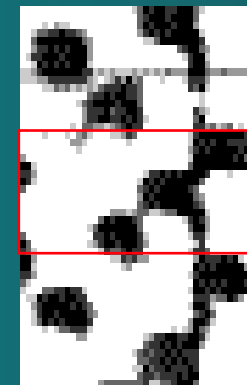
A G C T



C  
A Gln30  
G  
A Ile31  
T  
C  
T  
A  
C Tyr32

K31S

A G C T

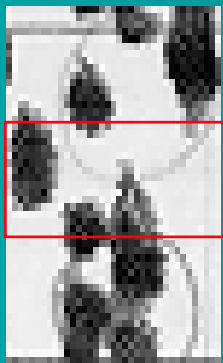


C  
A Gln30  
G  
T Ser31  
T  
C  
G  
T  
A  
C Tyr32

# Results: DNA sequence of Lys131-mutants

WTAK

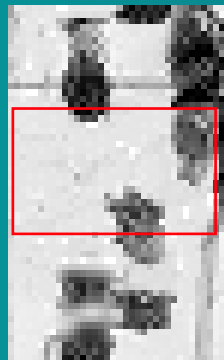
AGCT



C  
T  
G Leu130  
A  
A Lys131  
G  
C  
C Arg132  
C

K131F

AGCT



C  
T  
G Leu130  
T  
T Lys131  
C  
C Arg132  
G  
C

K131A

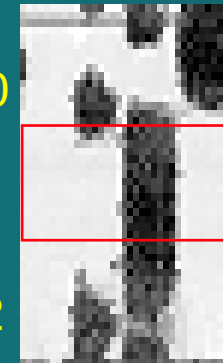
AGCT



C  
T  
G Leu130  
G  
G Lys131  
C  
C Arg132  
C  
G  
C

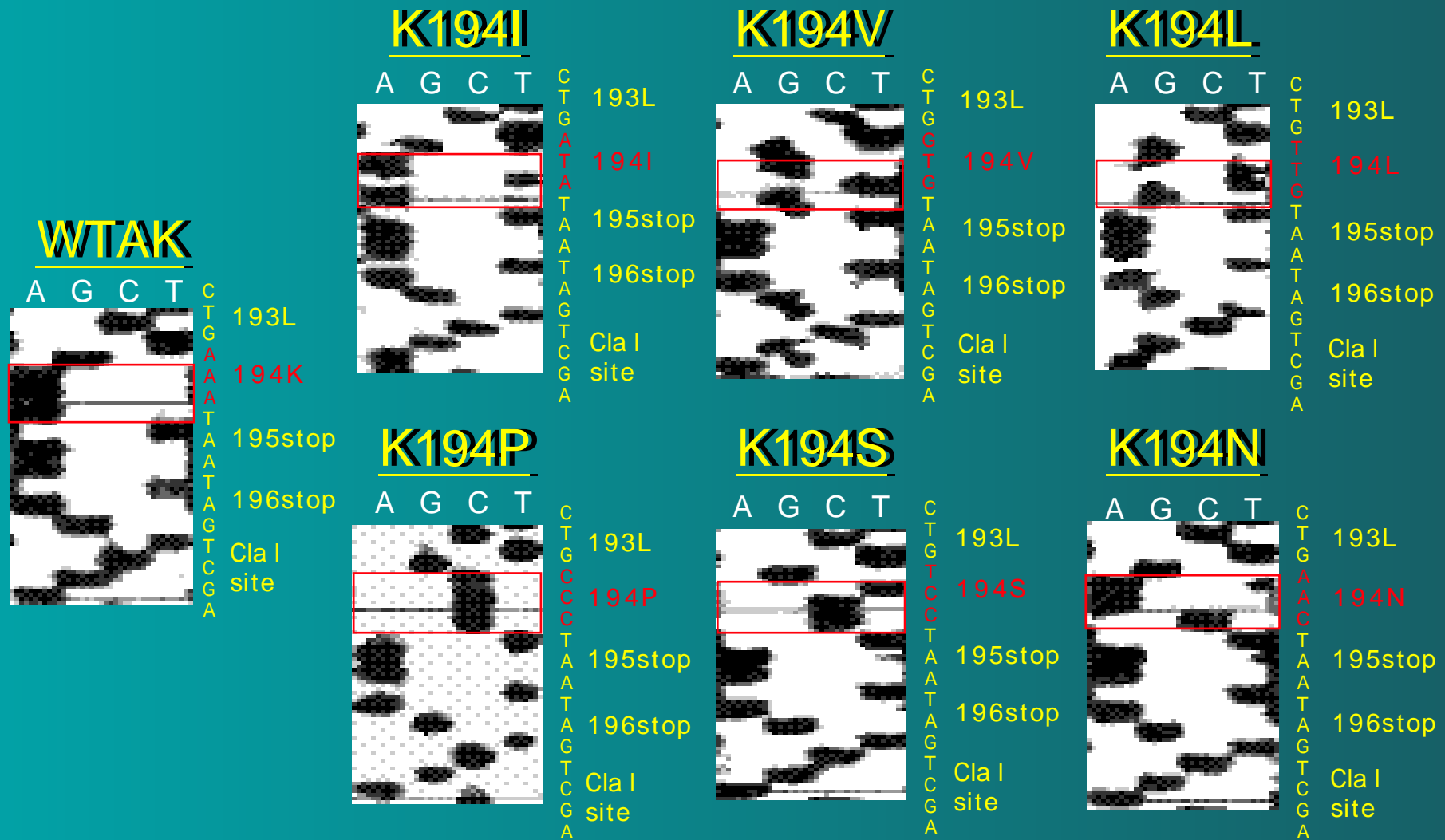
K131P

AGCT



C  
T  
G Leu130  
C  
C Lys131  
C  
C Arg132  
G  
C

# Results: DNA sequence of Lys194-mutants



## Results of site-directed mutagenesis

Target Residue	Mutagenesis Efficiency		Mutants
• Lys9 (AAG)	33.3%	(5/15) <sup>a</sup>	K9P(CCG) K9F(TTC) K9L(CTC) × 2 K9T(ACC)
• Lys21(AAA)	10%	(1/10)	K21P(CCG)
• Lys27(AAA)	33.3%	(4/12)	K27L(CTC) K27V(GTC) K27R(CGC) K27I(ATA)
• Lys31(AAA)	26.7%	(4/15)	K31I(ATC) K31S(TCG) K31F(TTC) × 2
• Lys63(AAA)	8.3%	(1/12)	K63F(TTC)
• Lys131(AAG)	30%	(3/10)	K131A(GCC) K131F(TTC) K131P(CCC)
• Lys194(AAA)	40%	(8/20)	K194S(TCC) × 3 K194N(AAC) K194V(GTG) K194I(ATA) K194P(CCC) K194L(TTG)

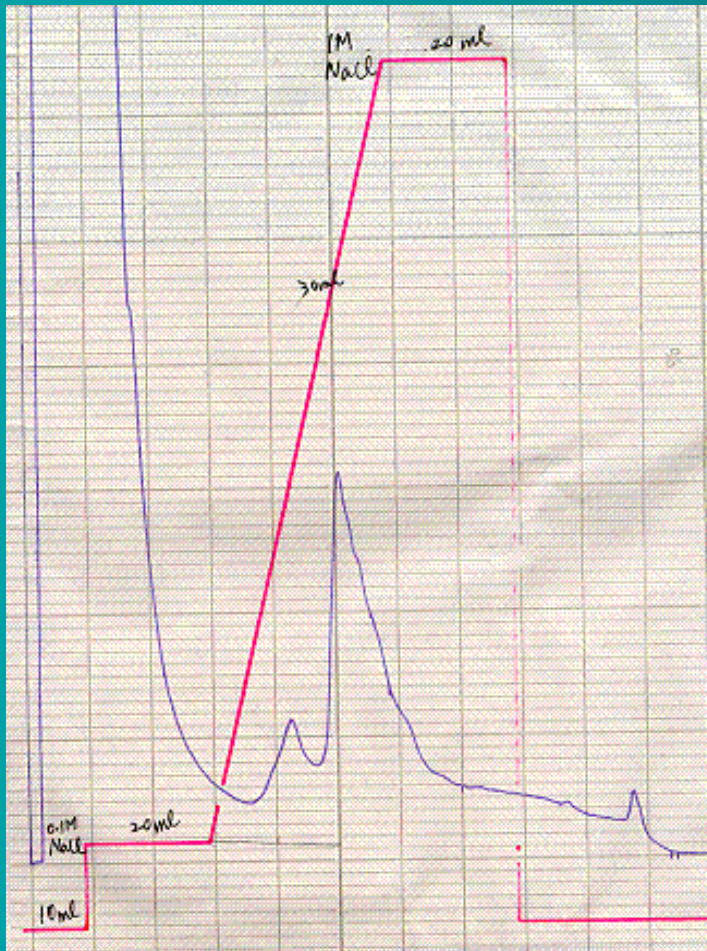
• <sup>a</sup> numbers in parenthesis represent ratio of the confirmed mutants/ screening number

## Protein expression and purification of wild-type and mutant AKs

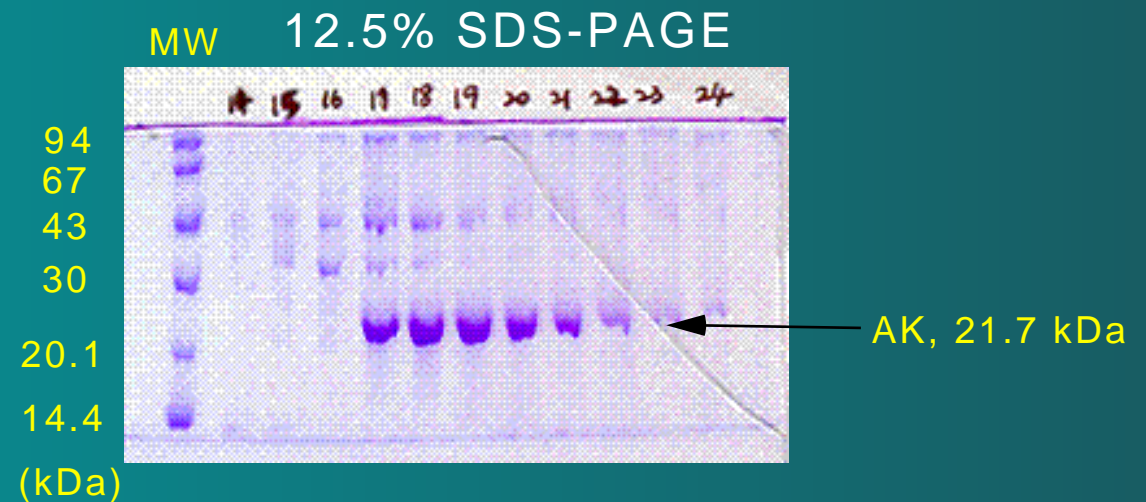
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- 1. Transformation with plasmid DNA and TG1 cells
- 2. Small culture of the cells in LB medium (5 ml ) overnight
- 3. Culture of the medium in 250 ml for 1 hr
- 4. Addition of isopropyl-  $\beta$ -D-thio-galactopyranoside (IPTG)  
(a final concentration of 1 mM)
- 5. Culture the medium for 16 hr
- 6. Centrifugation of the medium (5,000 X g for 20 min)
- 7. Disruption of the pellet of E.coli cells in standard buffer  
(10ml) (Ultrasonicator, 20kHz, 20W, 3min)
- 8. Centrifugation of the homogenate (12,000 X g for 20 min)
- 9. Blue sepharose CL-6B column chromatography
- 10. 12.5% SDS-PAGE and concentration
- 11. Gel filtration (Superose 12)
- 12. 12.5% SDS-PAGE
- 13. Measurement of the concentration of protein (Lowry method)
- 14. Kinetic analysis of forward reaction of AK

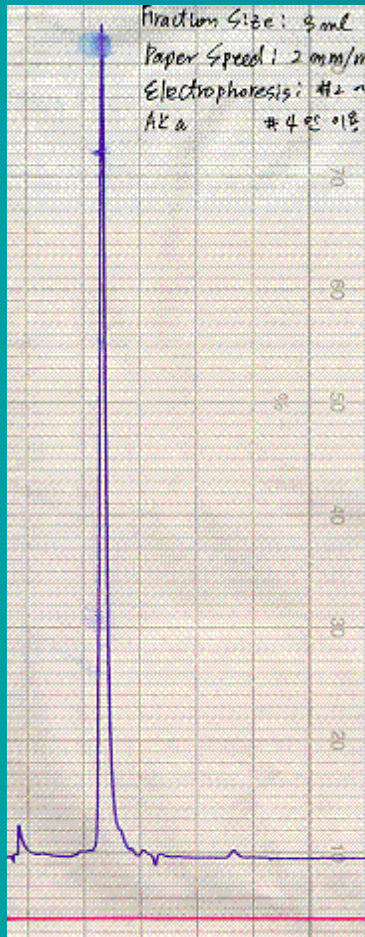
# Blue Sepharose Chromatography



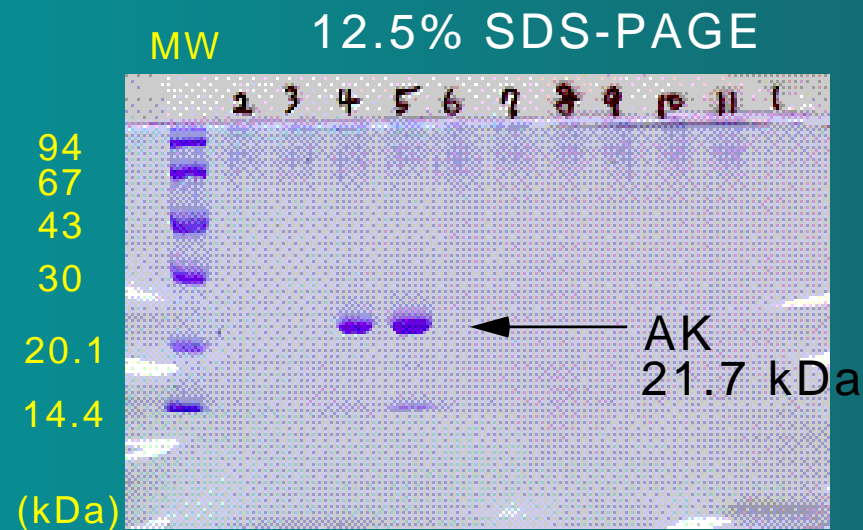
- Column: Blue Sepharose CL-6B ( 1 X 5 cm)
- Standard buffer: 20mM Tris-HCl, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4
- Gradient: 0 - 1 M NaCl
- Velocity: 0.5 ml/min
- Fraction size: 3 ml



# Superose 12 Column Chromatography



- Column: Superose 12 ( 1 X 30 cm )
- Imidazole buffer: 5mM imidazol-HCl, 1 mM EDTA, 0.1 mM dithiothreitol, pH 6.9
- Velocity: 0.5 ml/min
- Fraction size: 2 ml
- 



## Results of protein purification

Target Residue	Mutagenesis Efficiency	Mutants	Protein Yield (mg)	(% of wild-type AK)	AK Activity
Lys9 (AAG)	33.3% (5/15) <sup>a</sup>	K9P(CCG)	1.60	(23%) <sup>b</sup>	1.2 <sup>c</sup>
		K9F(TTC)	1.93	(28%)	0.5
		K9L(CTC) × 2	0.92	(13%)	8.0
		K9T(ACC)	0.31	(4%)	40.4
		K21P(CCG)	0.16	(2%)	5.1
Lys21(AAA)	10% (1/10)	K27L(CTC)	1.60	(23%)	4.2
Lys27(AAA)	33.3% (4/12)	K27V(GTC)	insoluble		-
		K27R(CGC)	0.52	(8%)	2.3
		K27I(ATA)	5.44	(79%)	0.4
Lys31(AAA)	26.7% (4/15)	K31I(ATC)	2.03	(29%)	0.7
		K31S(TCG)	1.88	(27%)	0.5
		K31F(TTC) × 2	1.69	(24%)	6.9
Lys63(AAA)	8.3% (1/12)	K63F(TTC)	0.94	(14%)	0.7
Lys131(AAG)	30% (3/10)	K131A(GCC)	2.07	(30%)	1.9
		K131F(TTC)	3.95	(57%)	1.3
		K131P(CCC)	insoluble		-
		K194S(TCC) × 3	1.04	(15%)	12.6
Lys194(AAA)	40% (8/20)	K194N(AAC)	0.89	(13%)	4.3
		K194V(GTG)	1.85	(27%)	1.7
		K194I(ATA)	1.88	(27%)	0.3
		K194P(CCC)	5.26	(76%)	0.2
		K194L(TTG)	1.26	(18%)	1.0

- <sup>a</sup> numbers in parenthesis represent ratio of the confirmed mutants/ screening number
- <sup>b</sup> numbers in parenthesis represent % of wild-type AK; the yield of wild-type AK was 6.90 mg.
- <sup>c</sup> represents % of wild-type AK. Each mutant was assayed in a forward reaction at a fixed concentration of 1mM of MgATP and AMP.







## Kinetic results of Lys-mutants

Residue	mutant	Km MgATP	Km AMP	kcat	kcat/Km MgATP	kcat/Km AMP	
Lys9	K9P	-	-	- -	-	-	
	K9F			- - -	- - -	- - -	
	K9L		+	- - -	- - -	- - - -	
	K9T	+ +	+ +	- -	- - -	- - -	
Lys21	K21P	+ + +	+ +	- -	- - -	- - -	
Lys27	K27R			- -	- - -	- -	
	K27L	+		- -	- - -	- -	
	K27I	+		- - -	- - - -	- - -	
Lys31	K31F	+ +		- -	- - - -	- - -	
	K31I	-		- -	- - -	- -	
	K31S	+	+ + + +	- - -	- - -	- - - -	
Lys63	K63F	+	-	- - -	- - - -	- -	
Lys131	K131A	+ + +		- - -	- - - -	- - -	
	K131F			- - -	- - -	- - -	
Lys194	K194S	+ +		-	- -	-	
	K194I	-	-	- -	-	- -	
	K194L	+	-	-	- -	-	
	K194P		-	- - -	- - -	- -	
	K194N		-	- -	- -	- -	
	K194V	-	+ + + +	- -	- -	- - - -	
Kmの減少 Kmの増加	Km < 1.0	-		kcat	10 %	- 100 %	-
	1.0 < Km < 5.0	空欄			1 %	- 10 %	- -
	5.0 < Km < 10.0	+			0.10 %	- 1 %	- - -
	10.0 < Km < 15.0	+ +				< 0.10 %	- - - -
	15.0 < Km < 20.0	+ + +					
	20.0 < Km < 25.0	+ + + +					

## まとめ

- (1) ヒトAKをコードするcDNAを用い、比較的容易に短時間に複数の変異体を得る大腸菌発現系を確立した。
- (2) リジン残基(K9, K21, K27, K31, K63, K131, K194)に対して、ランダムに部位特異的変異導入を行い、26種類の変異型酵素を得た。
- (3) 変異型酵素の酵素キネティクス解析により、基質(ATP, AMP)親和性及び触媒作用の増加や減少が観察され、リジン残基は、酵素活性に必須なアミノ酸残基であることが示唆された。