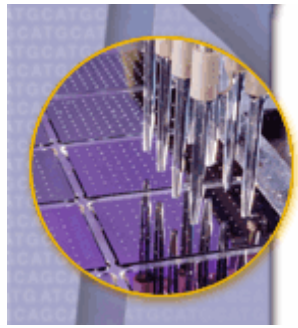
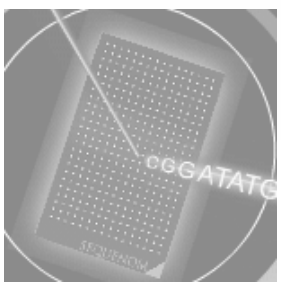


Diagnosis of human genetic mutations based on DNA microarray technology

Hyun Gyu Park



Department of Chemical and Biomolecular Engineering

KAIST

Outline

I. Introduction

II. Oligonucleotide chip for the Diagnosis of *HNF-1α* mutations

III. Array-based mutation detection of the *BRCA1* using direct probe/target hybridization

IV. Detection of mutations in *BRCA1* using SBE reaction and zip-code microarray

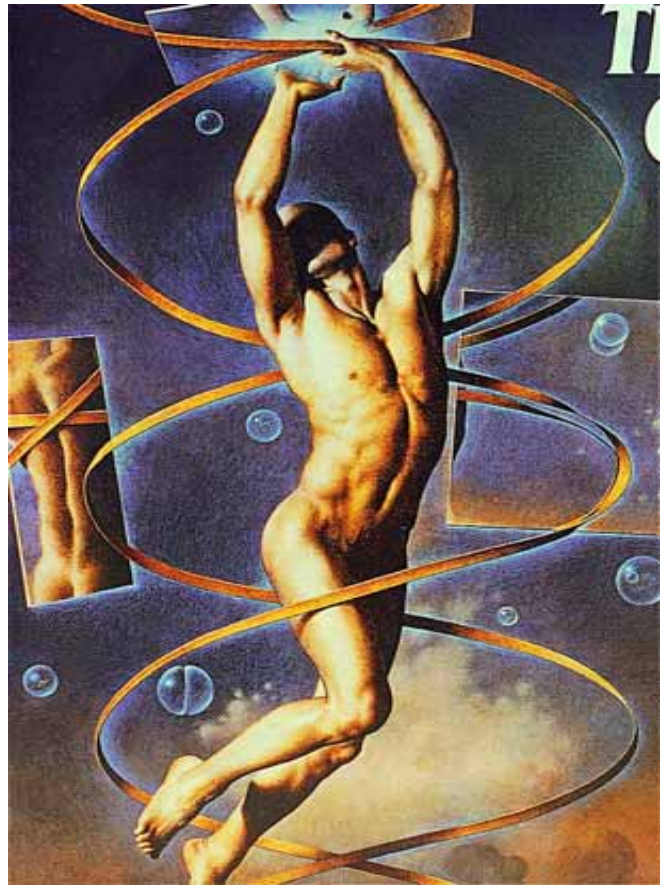
V. Diagnosis of *HNF-1α* mutations on a PNA zip-code microarray by single base extension

VI. Summary

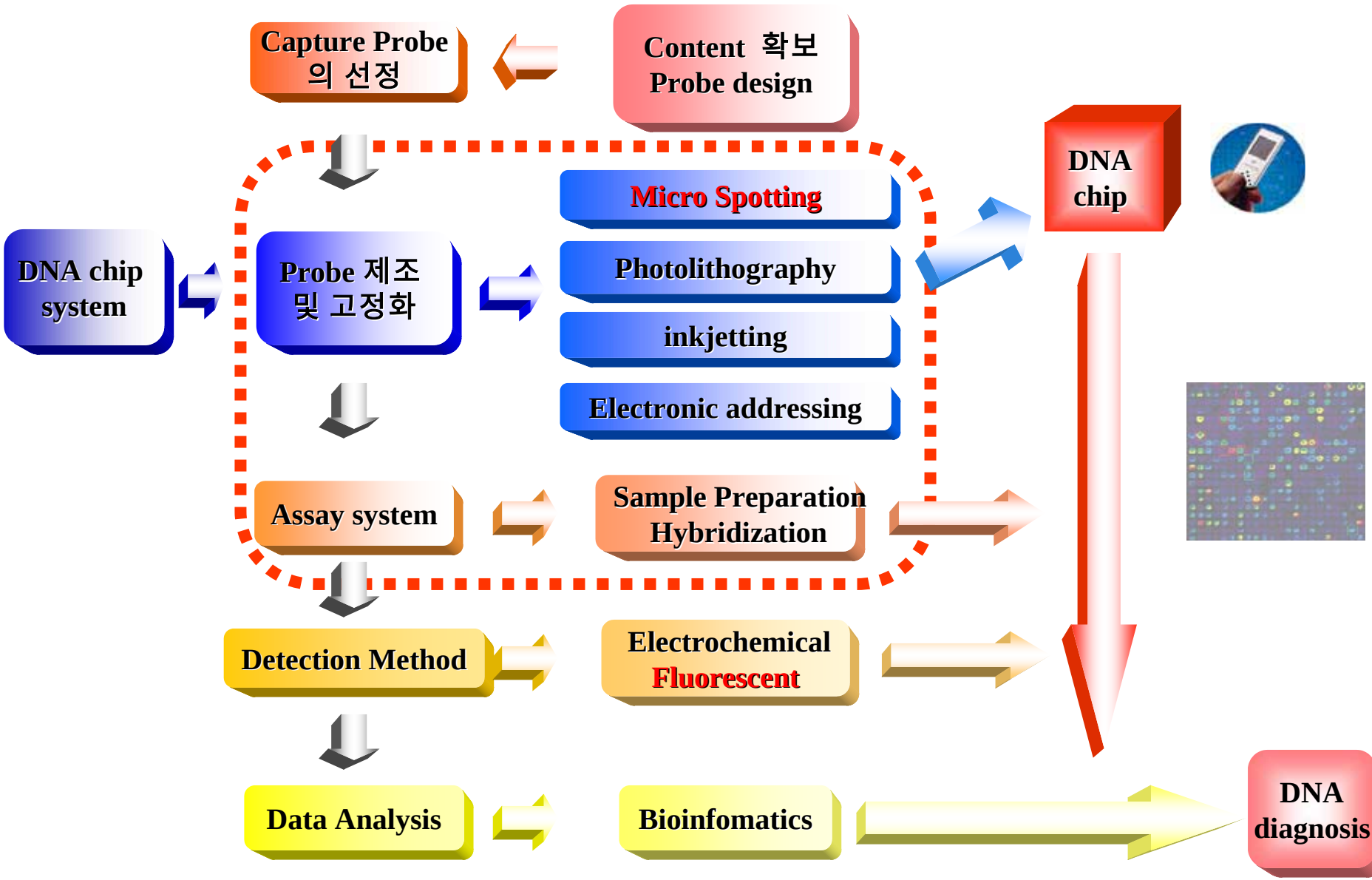
❑1990년 시작
❑ 2001년 2월 초안 발표 (Science & Nature)
❑ 2003년 4월15일: 완성 발표 (99.9%)







❑ 30억개의 염기 배열 결정 (A, T, G, C)
❑ 인간 유전자의 파악: 10만개 → 3만개 내외
❑ 초파리나 미개한 기생충인 선충의 불과 2배의 유전자 수
❑ 현재 인체에서 기능하는 단백질의 종류: 20만개-30만개로 예상 (Craig Venter: 250,000)
- 하나의 유전자가 평균 10종류의 단백질 발현에 관여
❑ 여러 유전자들이 coupling되어 여러 종류의 단백질을 발현 가능성


Capture Probe 의 선정
Content 확보 Probe design
DNA chip system
Probe 제조 및 고정화
Micro Spotting
Photolithography
inkjetting
Electronic addressing
DNA chip
Assay system
Sample Preparation Hybridization
Detection Method
Electrochemical
Fluorescent
Data Analysis
Bioinfomatics
DNA diagnosis

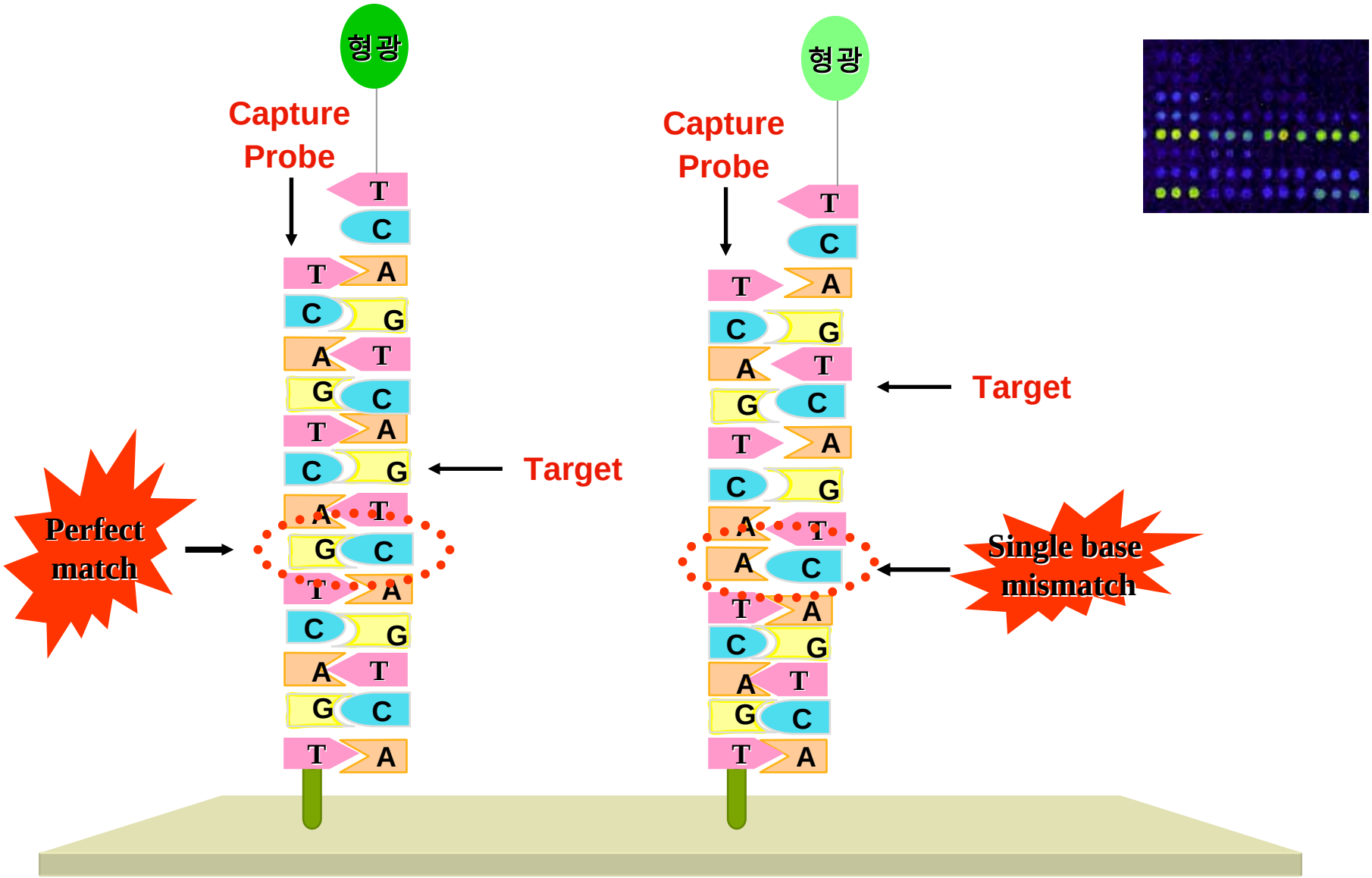
Oligonucleotide chip for the Diagnosis of *HNF-1α* mutations

MODY?

- Maturity Onset Diabetes of the Young
- Main feature
  - Diabetes often develops before the age of 25
  - Diabetes runs in families from one generation to the next
  - Diabetes may be treated by diet or tablets and does not always need insulin treatment
- Six gene related with MODY
  - **HNF-1α (MODY3) 70 % of MODY: chromosome 12 & 10 exons**
  - Glucokinase (MODY 2) 14 % of MODY
  - HNF-1β
  - HNF-4α
  - IPF1
  - Neuro D1

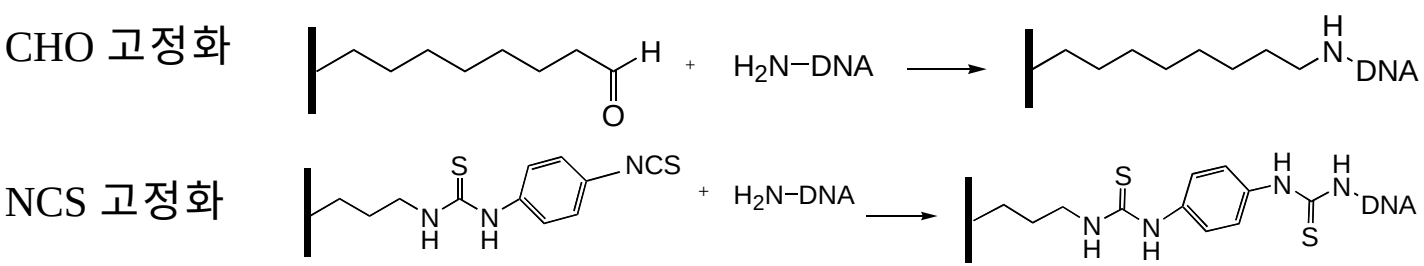
16 % of MODY

Direct capture/target hybridization



Materials and Methods

- Oligonucleotides (15 mer)
  - Capture probe: 5’ aminohexyl-terminated
  - Aldehyde and NCS glass
- Cy3-labeled RNA target probes
  - Promoter-tagged PCR from a wild-type blood sample
  - *In vitro* transcription of the PCR product (192-bp)
  - Sebsequent fragmentation with $MgCl_2$ (~50 nt)
- Advantages of RNA target over DNA target
  - Single-stranded
  - Better control for the size of the fragmented target probe



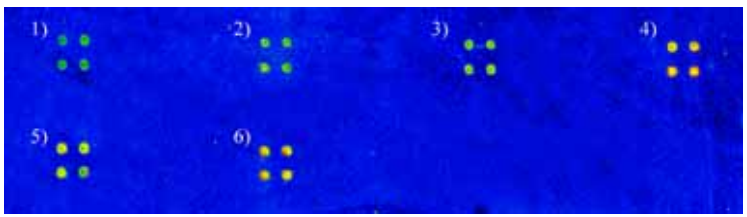
HNF-1α mutations represented on the MODY3 chip[a]

Probe number	Region	Sence sequence (5`↔3`)		Mutation effect	Position
		Wild-type	Mutant		
2-1	Exon 2	AAGTCCT**A**CCTGCAG	AAGTCCT**G**CCTGCAG	Y122C	1041
2-2	Exon 2	CCACAGC**G**GGAGGTG	CCACAGC**A**GGAGGTG	R131Q	1731
2-3	Exon 2	CCCACAG**C**GGGAGGT	CCCACAG**T**GGGAGGT	R131W	1942
2-4	Exon 2	AACCAGT**C**CCACCTG	AACCAGT**T**CCACCTG	S142F	2164
2-5	Exon 2	CAGAAGC**G**GGCCGCC	CAGAAGC**A**GGCCGCC	R159Q	2545
2-6	Exon 2	CAAGCAG**C**GAGAGGT	CAAGCAG**T**GAGAGGT	R171X	3033
3	Exon 2	GAGGAGC**G**AGAGACG	GAGGAGC**A**AGAGACG	R229Q	3459
4	Exon 2	CTCGTCA**C**GGAGGTG	CTCGTCA**T**GGAGGTG	T260M	3746

[a] The mutation site is underlined and in bold.

Optimization of immobilization buffer conditions

- **Capture probes: 5' aminohexyl terminated & labeled with 3' Cy3: direct evaluation of binding capacities**
- **Binding capacity: NCS > CHO**
- **Hybridization efficiency: CHO > NCS**

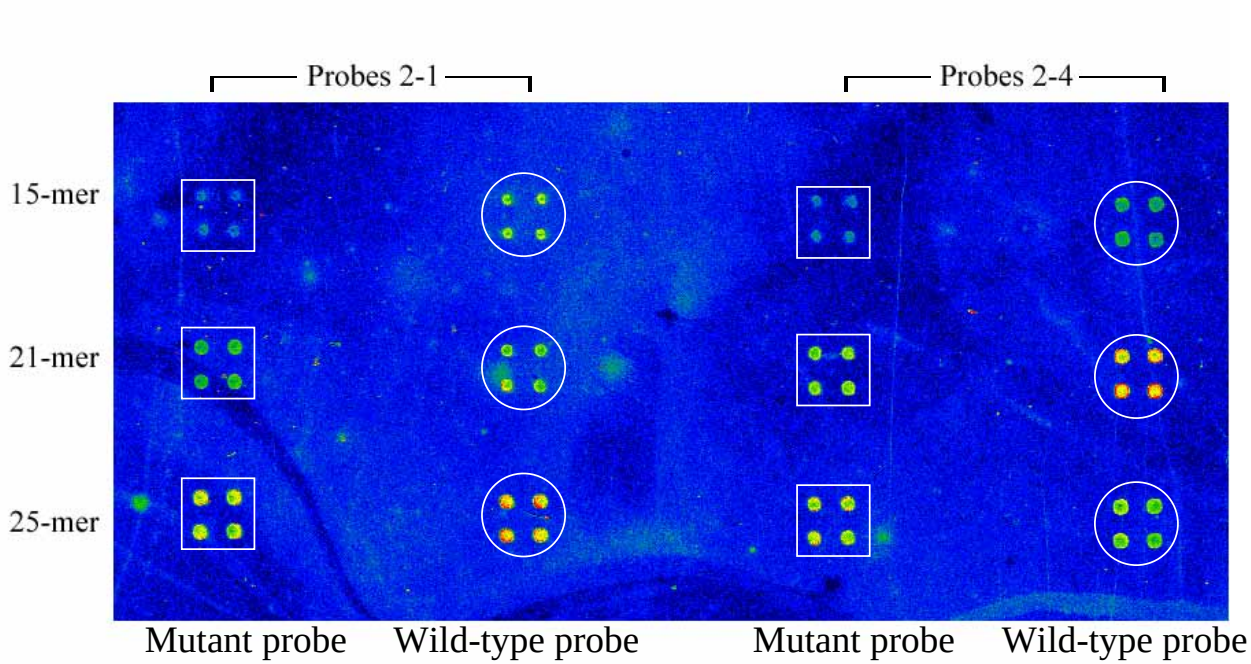


CHO-glass

NCS-glass

Buffer	CHO-glass	NCS-glass
Background signal	500	120
1) MES , pH 6.5	4200	4800
2) Distilled water	5200	7300
3) 3X SSC, pH 7.0	6000	12800
4) $NaHCO_3$, pH 9.0	7300	19500
5) $NaHCO_3$, pH 9.0	7800	16400
6) $NaHCO_3$, pH 9.0	7600	13900

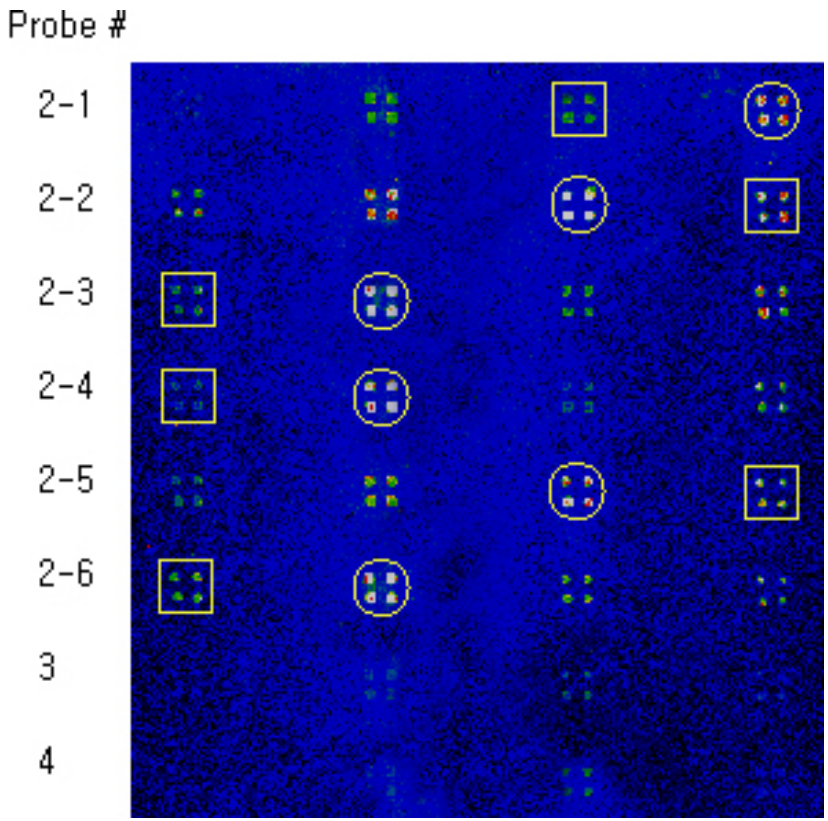
Effects of the capture probe length on the hybridized signal intensity and discrimination ratio



Length of capture probe	Relative signal intensity	Q_{pm}
15-mer	1	3.45
21-mer	1.26	2.23
25-mer	1.60	1.62

DNA chip analysis of mutations in exon2 of *HNF-1α*[a]

Probe number	Q_{pm}	Ratio of perfectly matched signal to negative control signals
2-1	7.4	7.5
2-2	1.7	1.8
2-3	4.5	3.2
2-4	7.6	7.4
2-5	1.8	3.8
2-6	2.3	2.9



Conclusions

- CHO-glass provides a more favorable environment for hybridization than NCS-glass, whereas the binding capacity of NCS-glass for amine-activated oligonucleotide is much greater than with CHO-glass
- Cy3-labeled RNA target probes by in vitro transcription of promoter-tagged PCR products from a wild-type blood sample & subsequent fragmentation
- We demonstrated that oligonucleotide chip-based analysis is a good candidate for routine clinical testing for *HNF-1α* mutations

Park et al. *Biosensors and Bioelectronics* (2005) in press.

Array-based mutation detection of *BRCA1* using direct probe/target hybridization

BRCA gene?

- Genetic susceptibility genes in breast and ovarian cancers.
- Approximately 10% of female breast cancer is due to inheritance of an altered, or mutated copy of BRCA1 (17q) or BRCA2 (13q).
- These mutations account for 75% of families with dominantly inherited breast and/or ovarian cancer.
- BRCA1: chromosome 17 & 24 exons
  - Length of cDNA: 5.6 kb
- BRCA2: chromosome 13 & 27 exons
  - Length of cDNA: 10.3 kb

Indirect fluorescence-labeling method

◆ Target sample

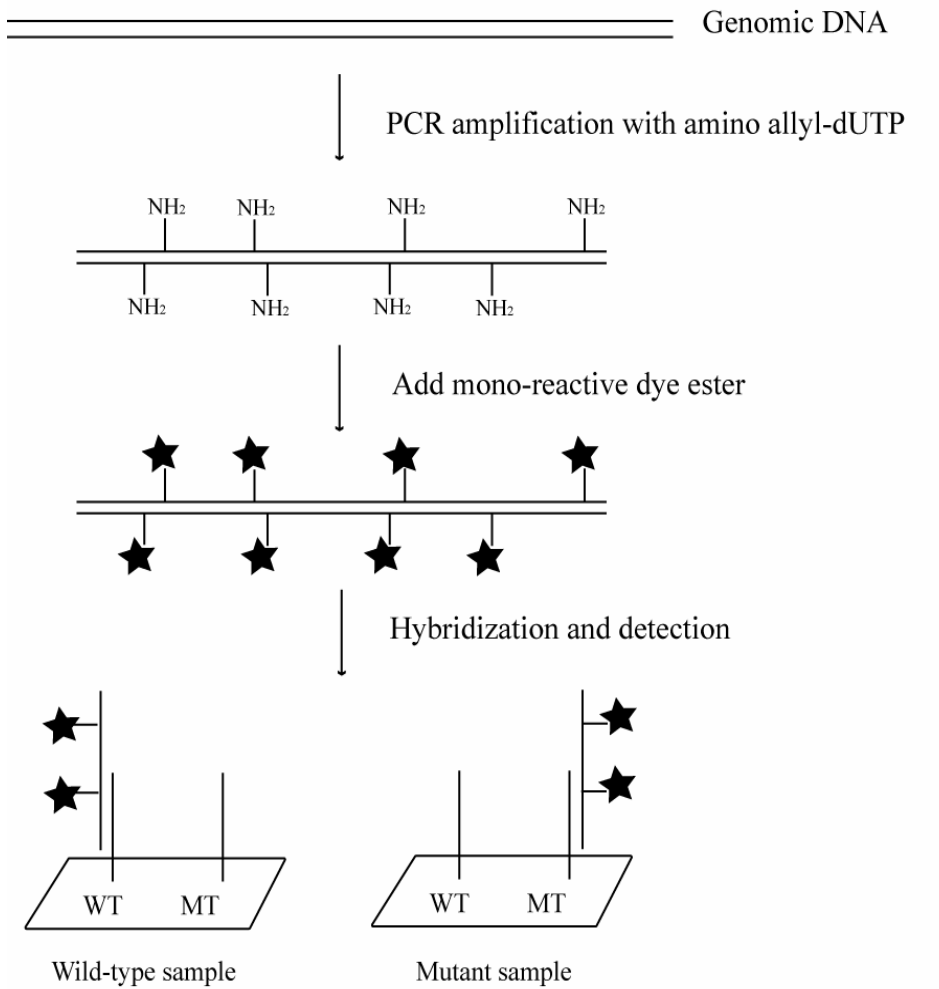
- Isolation of genomic DNA from whole blood of apparently healthy subject

- 6 mutlplex PCR amplification with aminoallyl-dUTP → cover 11 Korean specific mutations

- Monofunctional forms of Cy3 dye

◆ Indirect two-step mehtod

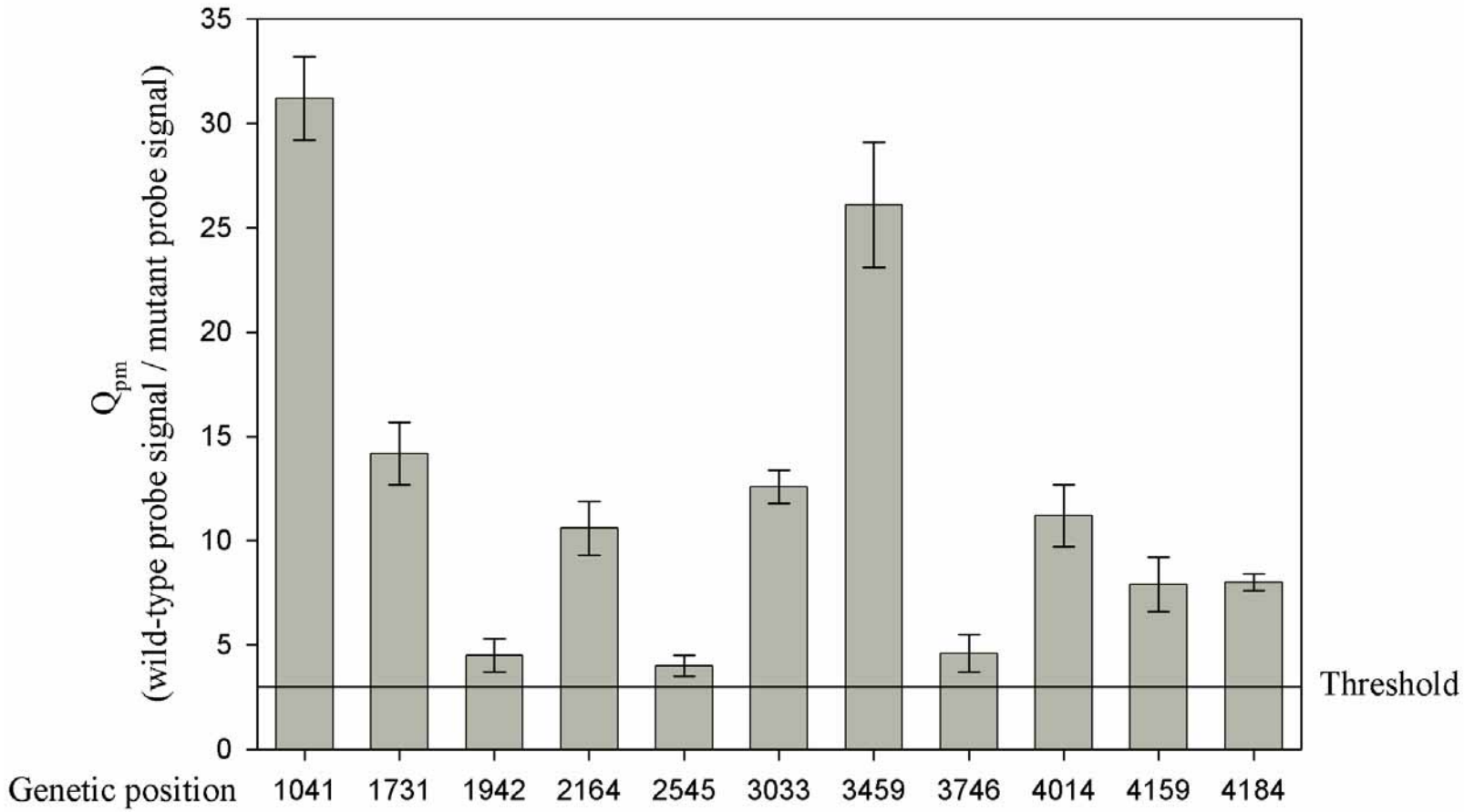
- Cost effective & better incorporation



Korean-specific 11 mutations in *BRCA1*

Region	Nucleotide change		Position
	Wild-type	Mutant	
Exon 11	AGC	T	1041
Exon 11	C	T	1731
Exon 11		Deletion A	1942
Exon 11	ACA	CC	2164
Exon 11		Deletion A	2545
Exon 11	G	T	3033
Exon 11	G	T	3459
Exon 11		Insertion A	3746
Exon 11	C	T	4014
Exon 11		Deletion GA	4159
Exon 11		Deletion TCAA	4184

Parallel detection of 11 mutation sites in *BRCA1* exon11 with multiplex PCR product from wild-type genomic DNA



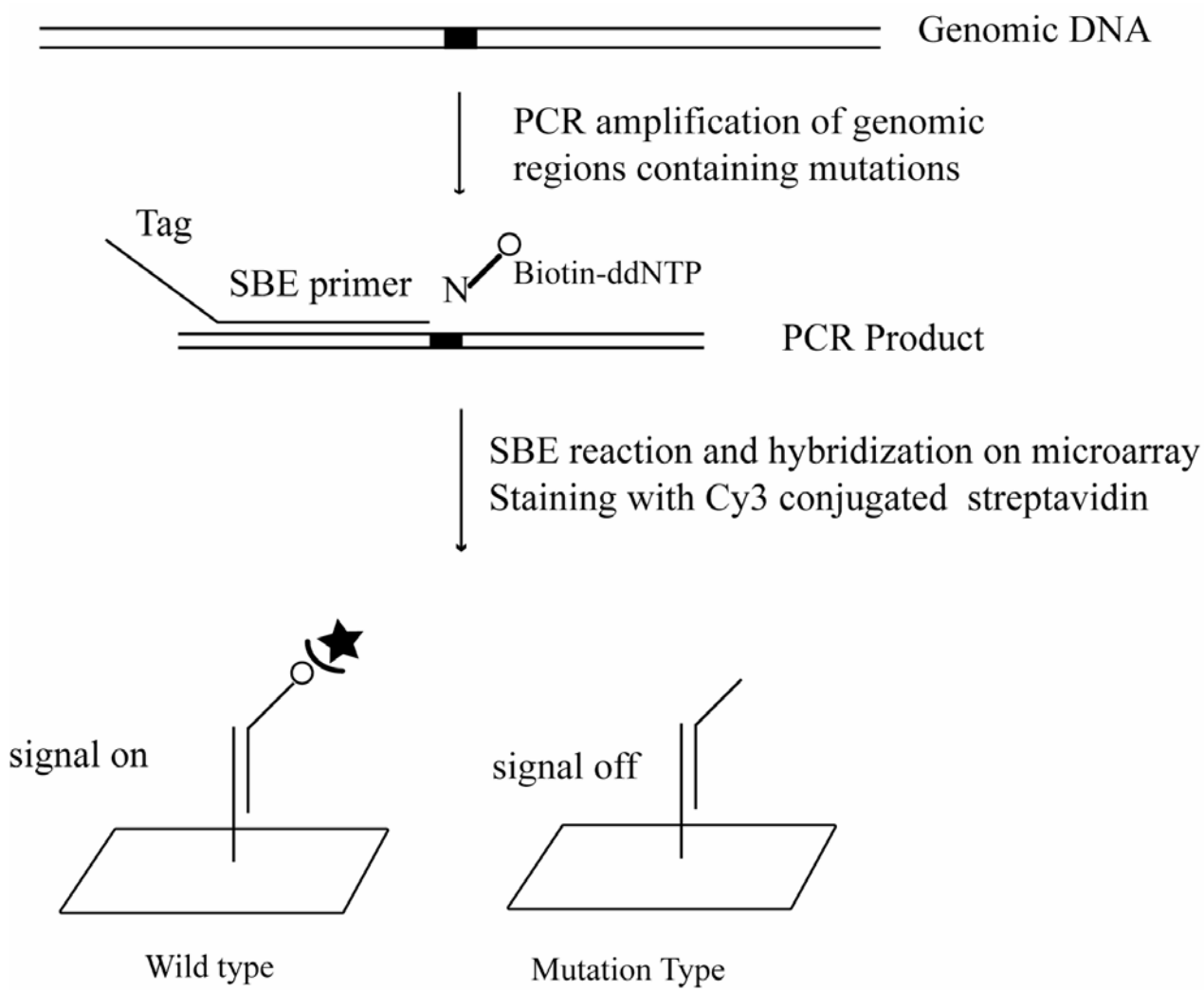
Conclusions

- We have developed a DNA microarray capable of detecting 11 mutations in *BRCA1*.
- Using the indirect two-step method to prepare the labeled PCR products, all of the 11 mutations were correctly genotyped.

Park et al. *Analytical Biochemistry*, **337**, 332-337, (2005)

Detection of mutations in *BRCA1* using SBE reaction and zip-code microarray

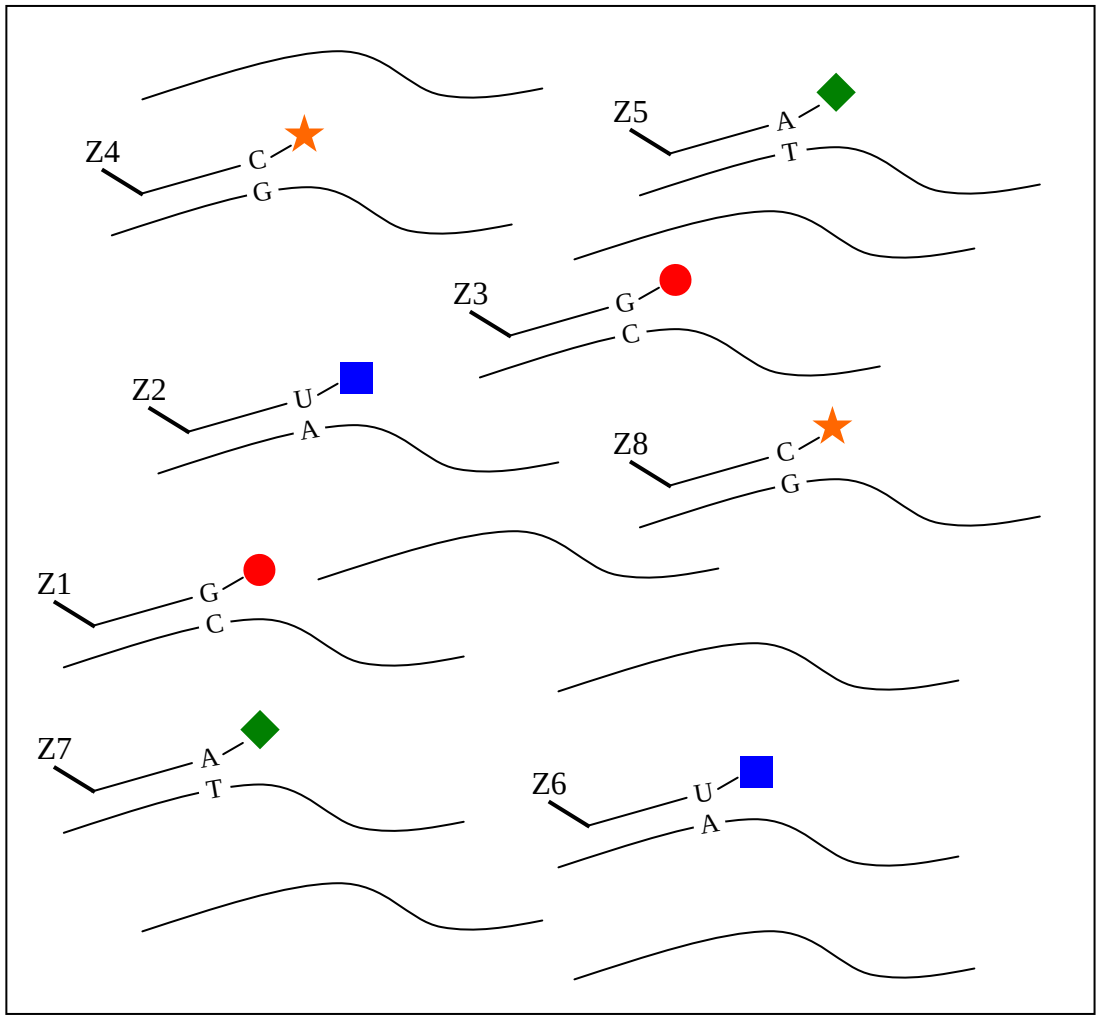
SBE & zip-code microarray

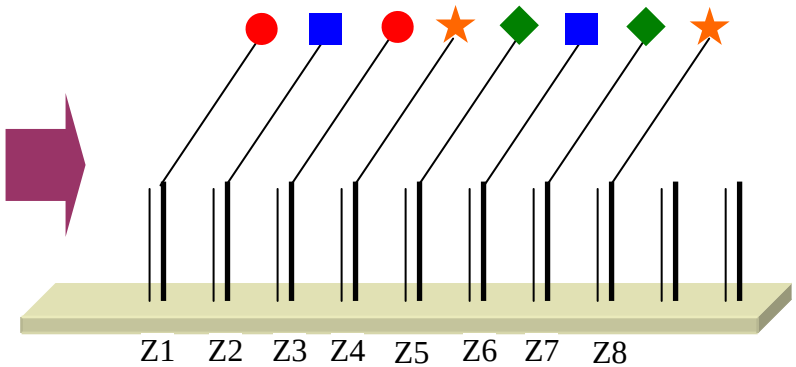


SBE with 4 different fluorescence-labeled dNTP & zipcode micoarray

ddATP ddCTP

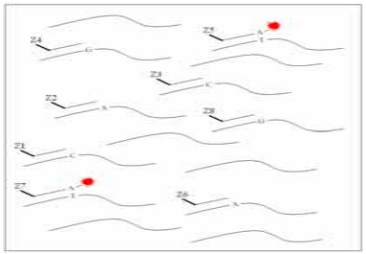
ddGTP ddUTP

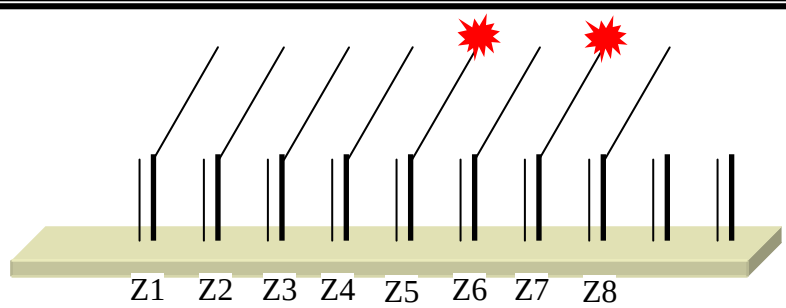




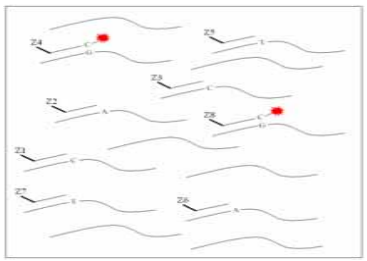
Four independent SBEs with each of four labeled dNTP

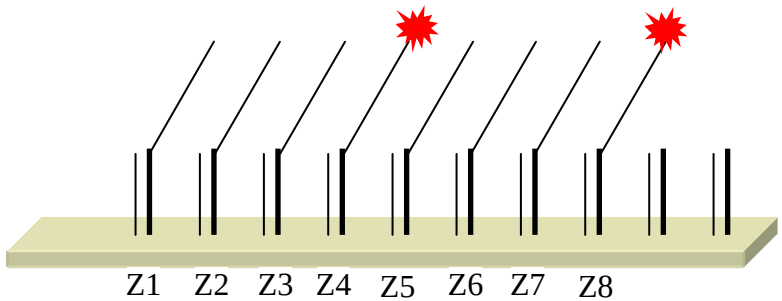
ddATP



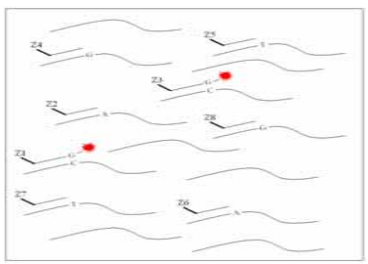


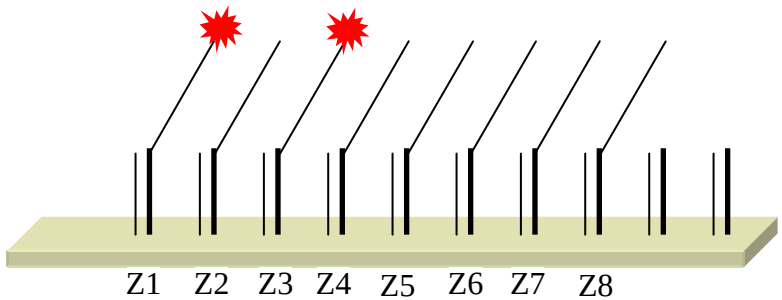
ddCTP



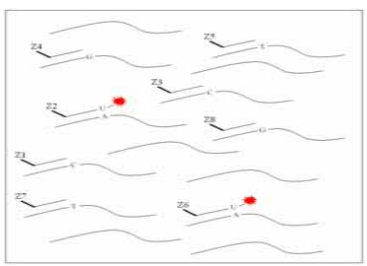


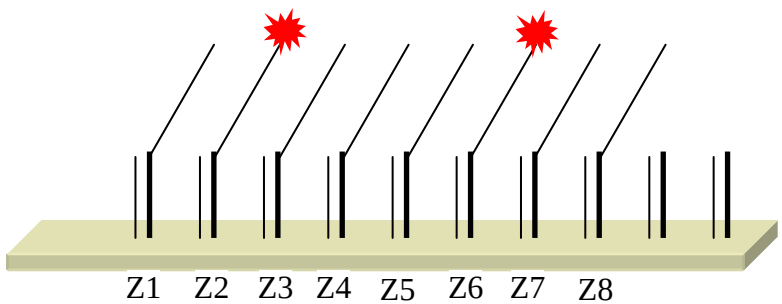
ddGTP





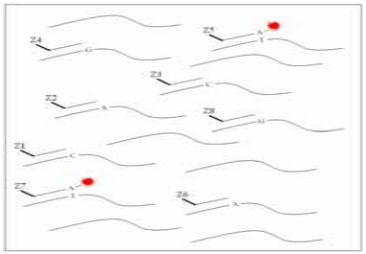
ddUTP

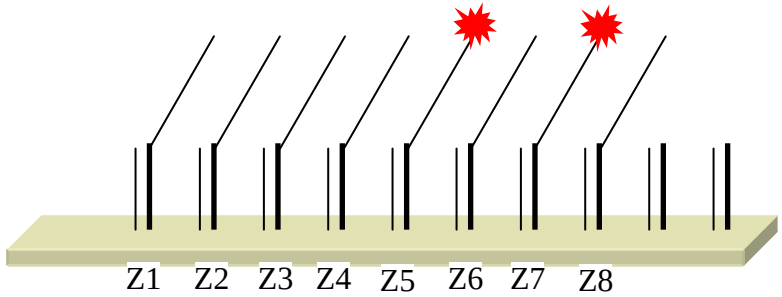




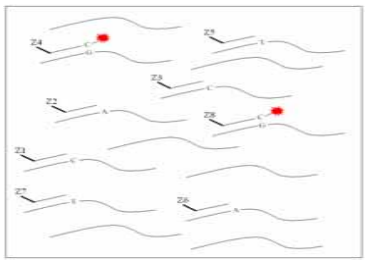
Four independent SBEs with each of four labeled dNTP & the other dNTPs

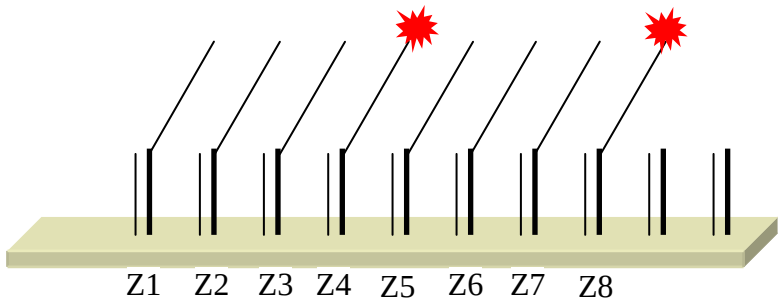
ddATP
ddCTP + ddGTP + ddUTP



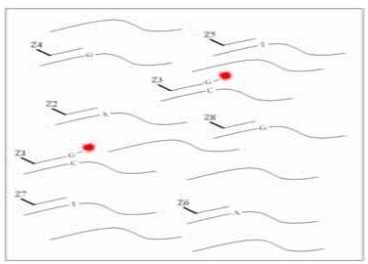


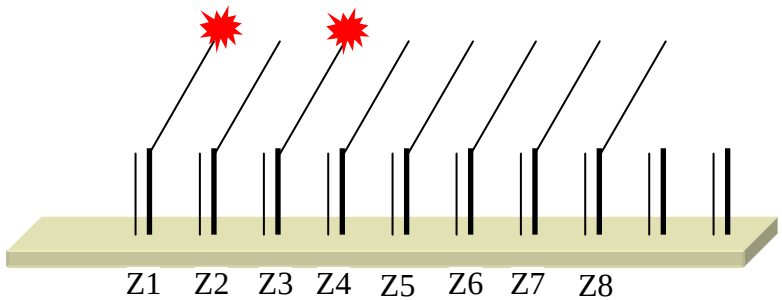
ddCTP
ddATP + ddGTP + ddUTP



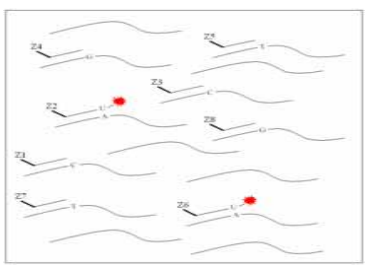


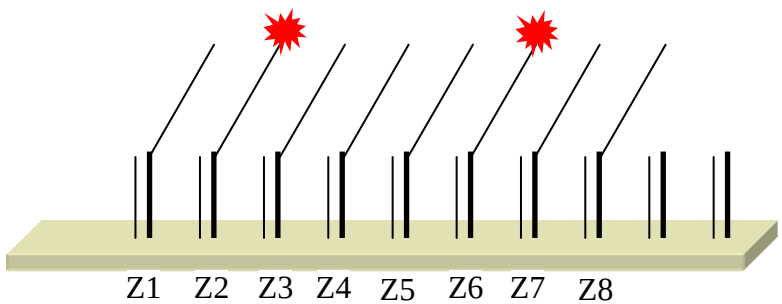
ddGTP
ddATP + ddCTP + ddUTP





ddUTP
ddATP + ddCTP + ddGTP

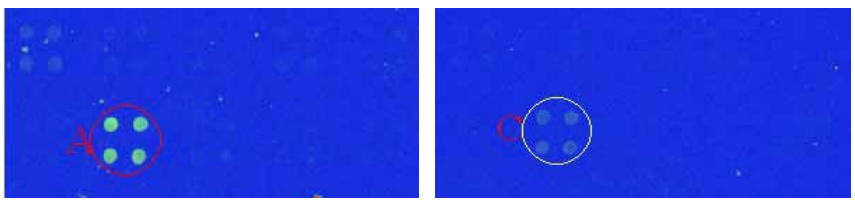




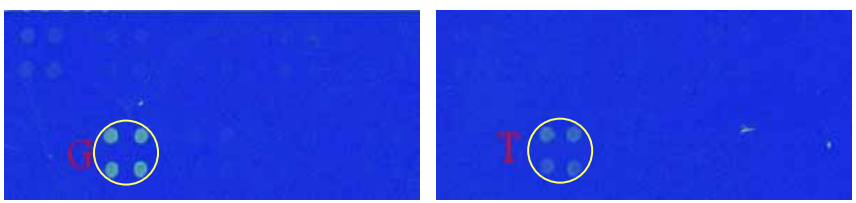
Non-specific incorporation of biotin-ddNTPs during SBE reaction by Thermosequenase

2545 site: **agtgtgcagcatttgaaaaccccaagggac**

ddNTP-biotin

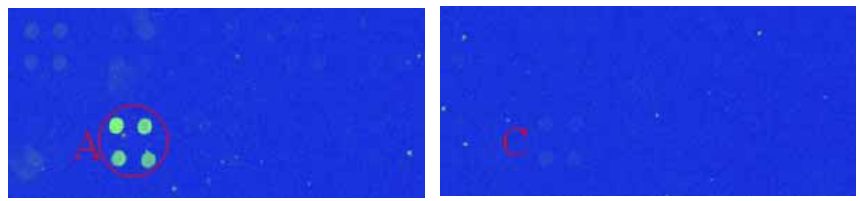


ddATP-biotin ddCTP-biotin

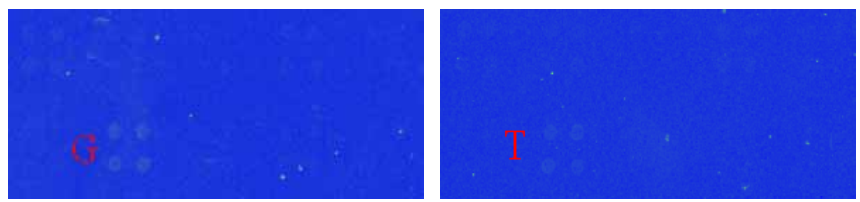


ddGTP-biotin ddUTP-biotin

ddNTP-biotin and unlabeled ddNTPs



ddATP-biotin ddCTP-biotin



ddGTP-biotin ddUTP-biotin

Korean-specific 7 mutations in *BRCA1* exon 11

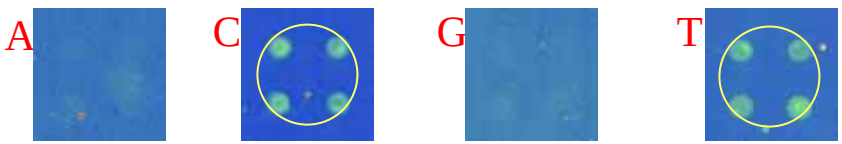
Region	Nucleotide change		Position
	Wild type	Mutant type	
Exon 11	C	T	1731
Exon 11		Deletion A	1942
Exon 11		Deletion A	2545
Exon 11	G	T	3033
Exon 11	G	T	3459
Exon 11		Insertion A	3746
Exon 11	C	T	4014

SBE reaction on zip-code microarray using heterozygote mutant DNA sample

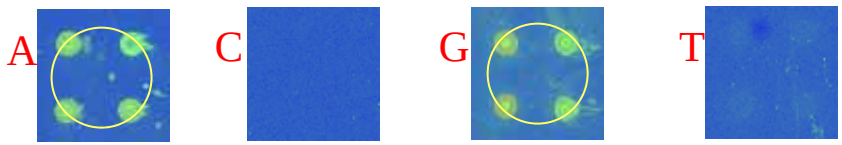
1731 Wild:	**agggaactaaccaaacggagcagaatggtc**
1731 Mutant:	**agggaactaactaaacggagcagaatggtc**
1942 wild:	**aagcacctaaaaagaataggctgaggagga**
1942 Mutant:	**aagcacctaaaagaataggctgaggagga**
2545 wild:	*agtgtgcagcatttg* **aaaaccccaagggac**
2545 Mutant:	*agtgtgcagcatttg* **aaaccccaagggac**
3746 wild:	**gaggggccaagaaattagagtcctcagaag**
3746 Mutant:	**gaggggccaagaaattaagagtcctcagaag**

SBE reaction on zip-code microarray using mutant DNA sample

1731 Heterozygote mutant (C/T)



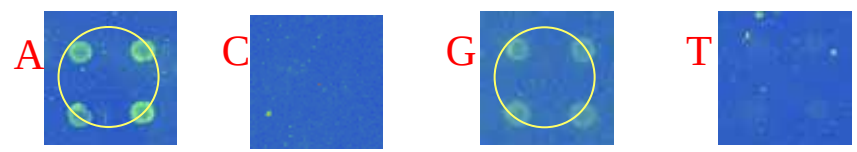
1942 Heterozygote mutant (A/G)



3746 Heterozygote mutant with single base (A) insertion

with 1st SBE primer (A/A)

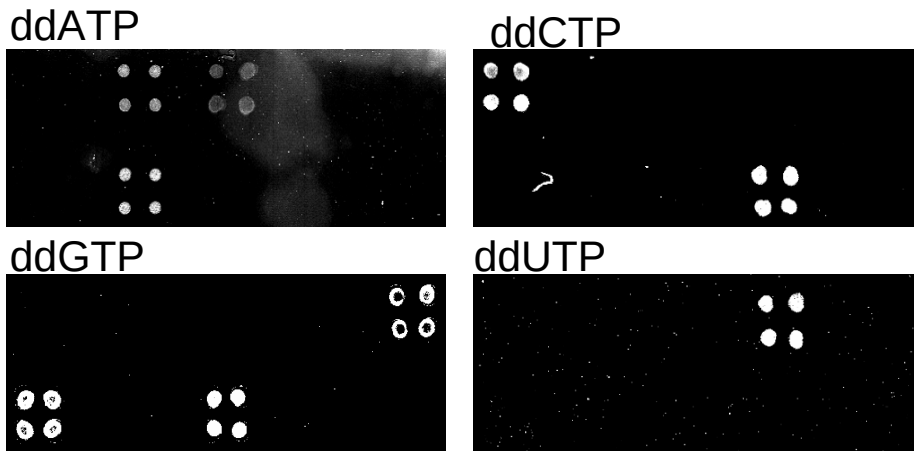
with 2nd SBE primer (A/G)



SBE reaction on zip-code microarray using mutant DNA sample

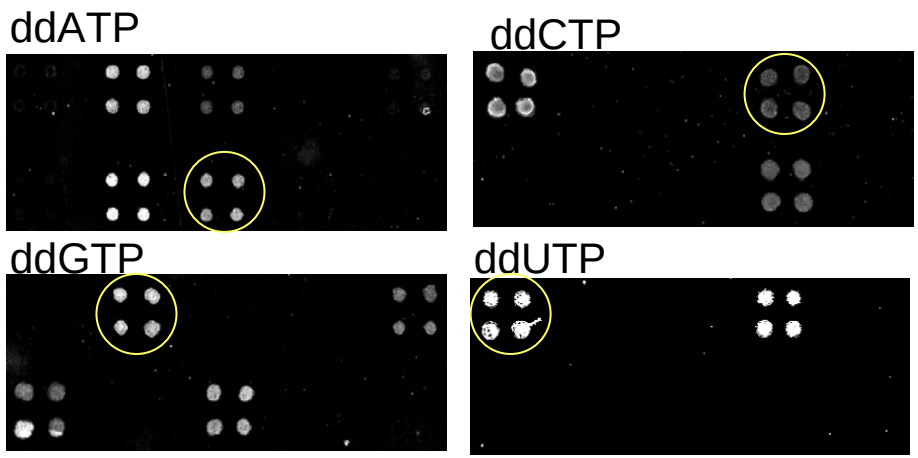
Wild-type sample

Zip1 01 (CC)	Zip2 02 (AA)	Zip3 03-1 (AA)	Zip4 03-2 (TT)	Zip5 04 (GG)
Zip6 05 (GG)	Zip7 06-1 (AA)	Zip8 06-2 (GG)	Zip9 07 (CC)	



Mixed sample with 4 mutants

Zip1 01 (CT)	Zip2 02 (AG)	Zip3 03-1 (AA)	Zip4 03-2 (TC)	Zip5 04 (GG)
Zip6 05 (GG)	Zip7 06-1 (AA)	Zip8 06-2 (GA)	Zip9 07 (CC)	



Conclusions

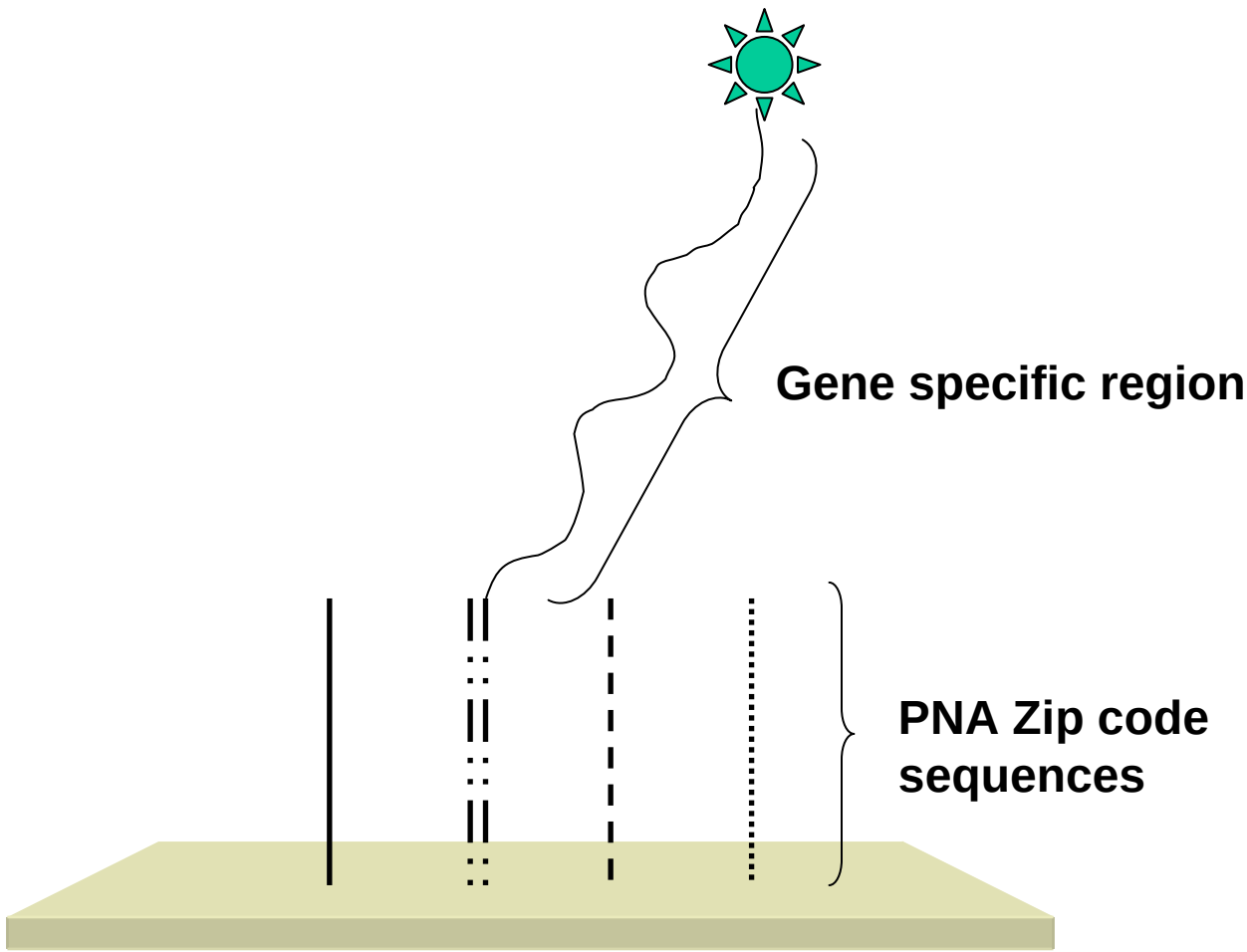
- **We have developed a multiplex assay to detect mutations in *BRCA1* by SBE reaction and zip-code microarray.**

- **All of the seven mutations were correctly genotyped using multiplex SBE reactions & zip-code microarray.**

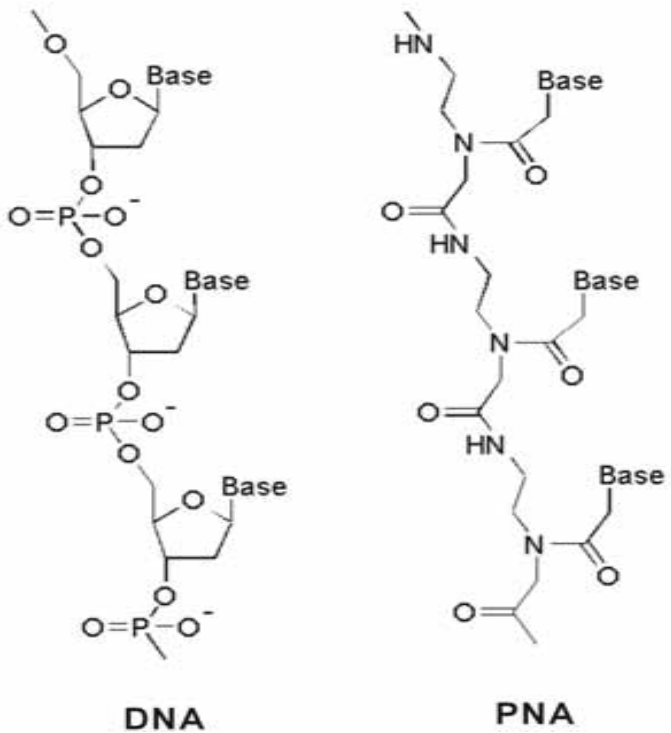
Park et al. Diagnosis of *BRCA* mutations on a zip-code microarray by SBE (2005) in preparation.

Diagnosis of *HNF-1α* mutations on PNA zip-code microarray by single base extension

PNA zip-code microarray

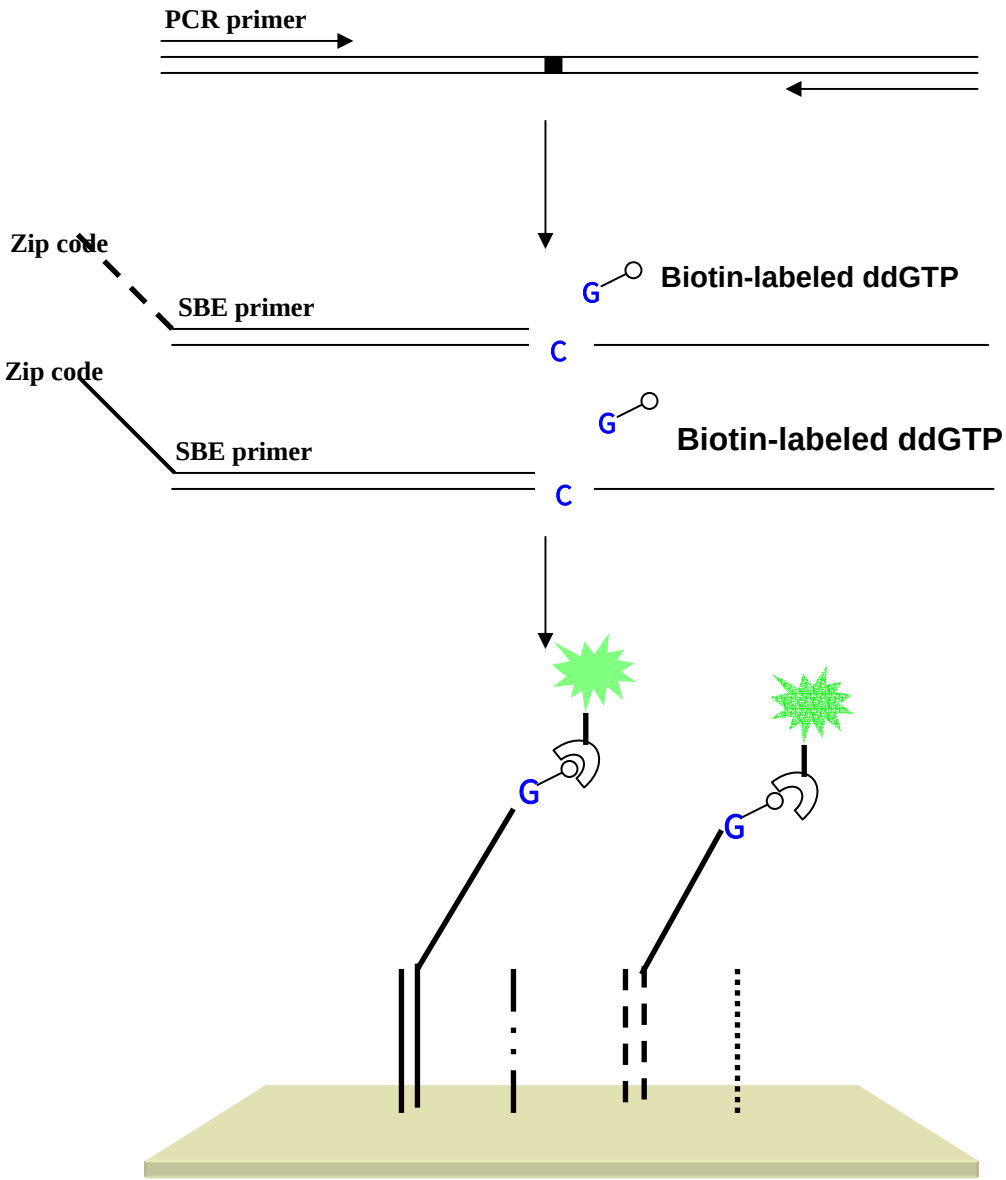


Peptide Nucleic Acid



	DNA	PNA
Hybridization affinity with DNA		At least 1 °C higher per base
Hybridization rate with DNA		100 - 5000 times faster
Required base length for diagnosis	20 - 30	11 - 15
T_m single mismatch	Lowering 10 °C	Lowering 15 °C
Chemical stability	Unstable in acid and base	Stable
Biological stability	Degradation by nuclease	Stable
Thermal stability	Moderate	Stable
Water solubility	Soluble	Restricted solubility
Cost	Low	High

Experimental Scheme



PCR amplification of target DNA

Conversion of double stranded PCR product to single stranded template

Multiplex SBE reaction with biotin labeled ddNTP in single tube

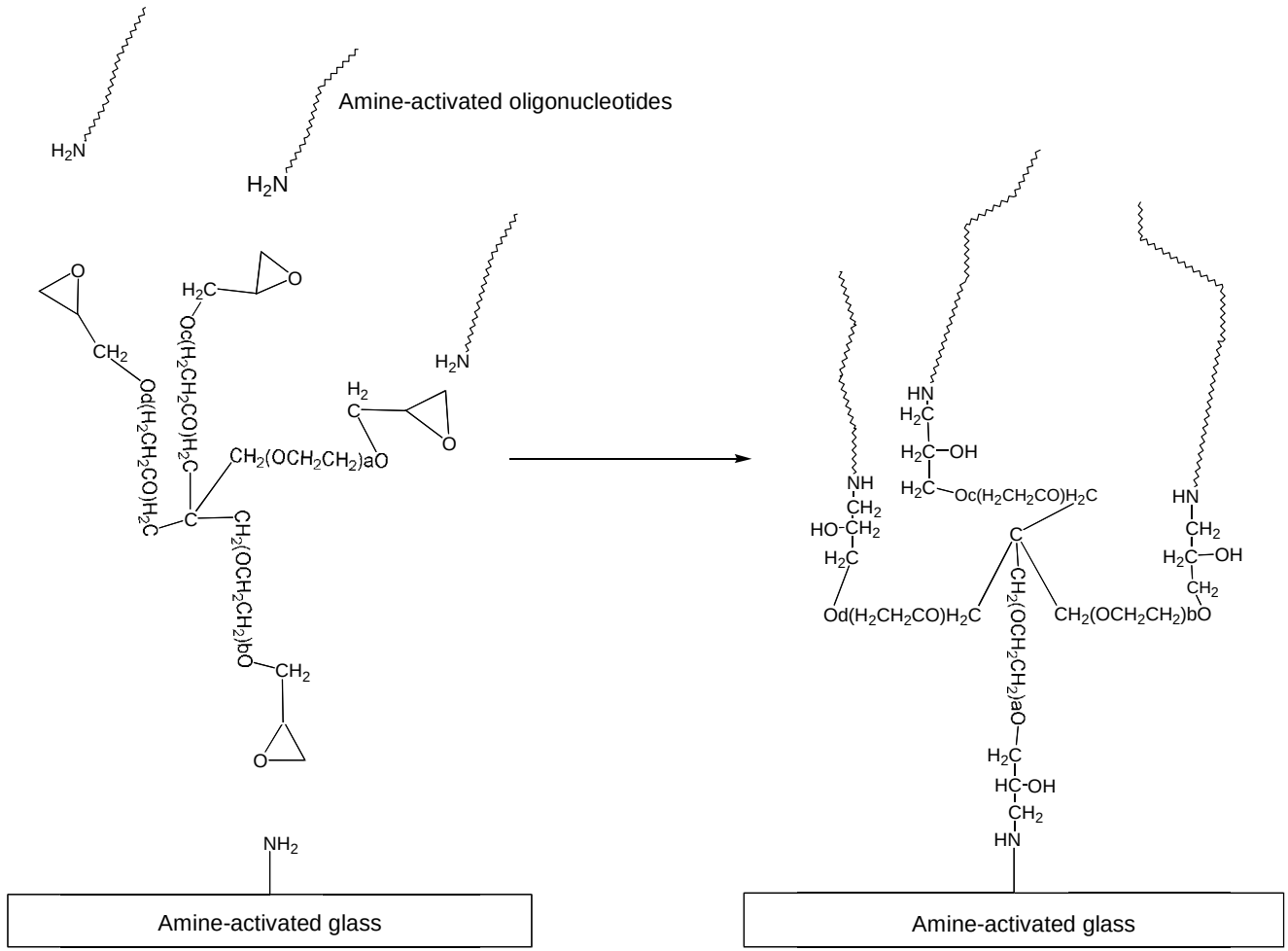
Hybridization of SBE reaction product on PNA microarray

Staining with
streptavidine-R-phycoerythrin

Epoxide-based oligonucleotide mmobilization

Epichlorohydrin + pentaerythritol ethoxylate ➡ linker compound with 4 epoxide groups

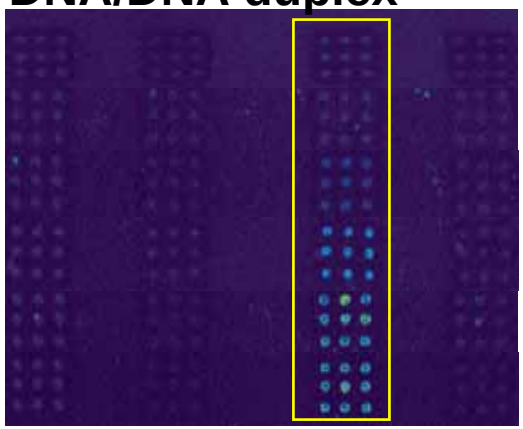
Greatly improved binding capacity of PNA zip-code molecules & reproducibility



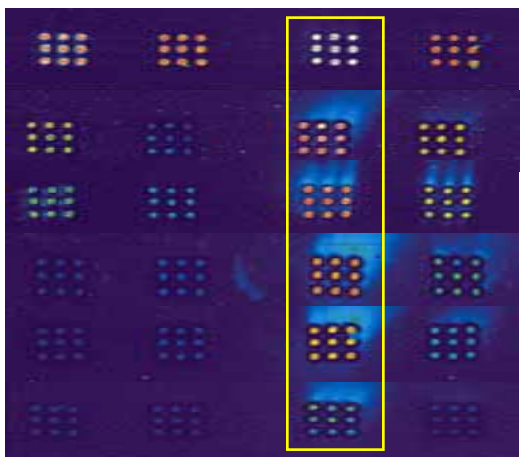
Effects of ionic strength on hybridization strength & specificity

쑥 1X SSPE

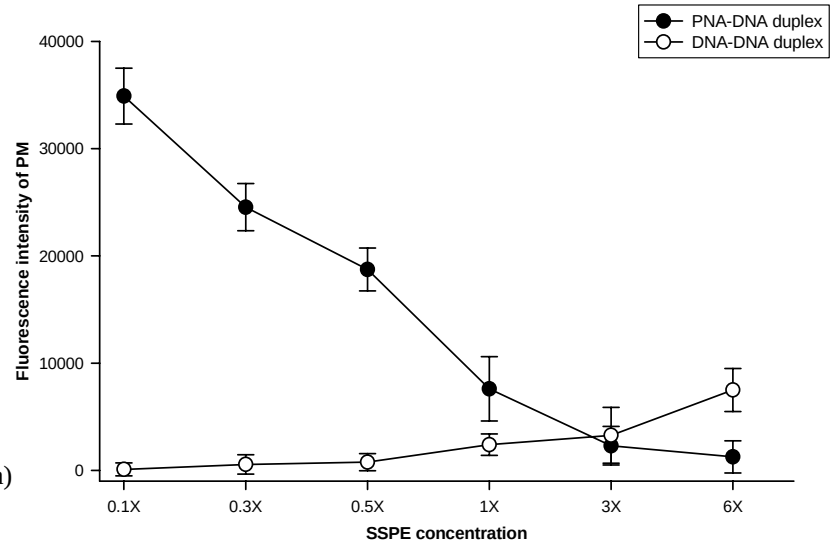
DNA/DNA duplex



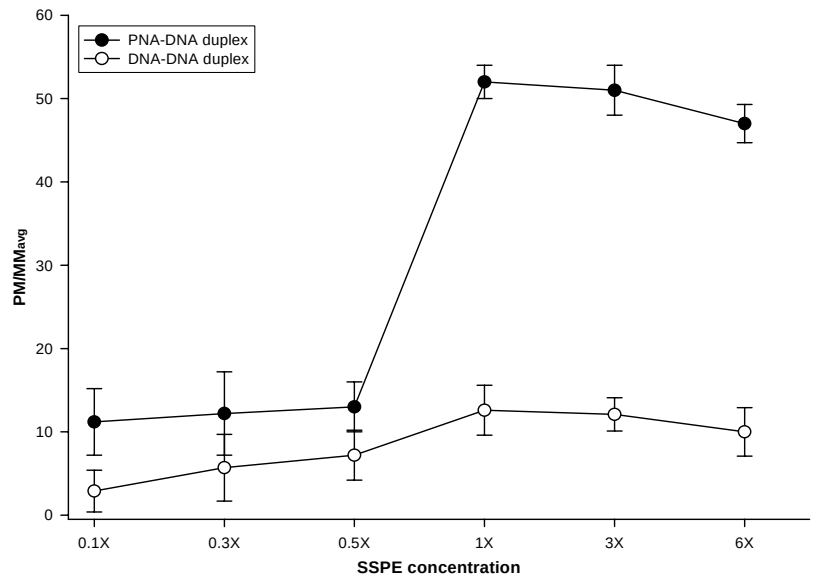
PNA/DNA duplex



(a)



(b)



Mutant information of exon2 of HNF-1α

Zip-code	Position	Wild-type	Mutation	Effect of Mutation
1	1821	C**G**G	C**A**G	R131Q
2	1814	C**C**C	C**A**C	P129T
3	1778	G**A**A	G**G**A	K117E
4	1810	G**C**G	G**T**G	R131W
5	1833	G**A**T	GT	D135fsdelA
6	1856	C**C**A	C**T**A	H143Y
7	1910	C**G**C	C**A**C	A161T
8	1869	C**A**C	C**G**C	H147R
9	1905	C**G**G	C**A**G	R159Q
10	1812	A**T**C	A**A**C	I128N

Primer sequences for the detection of mutations in exon 2 of HNF-1α

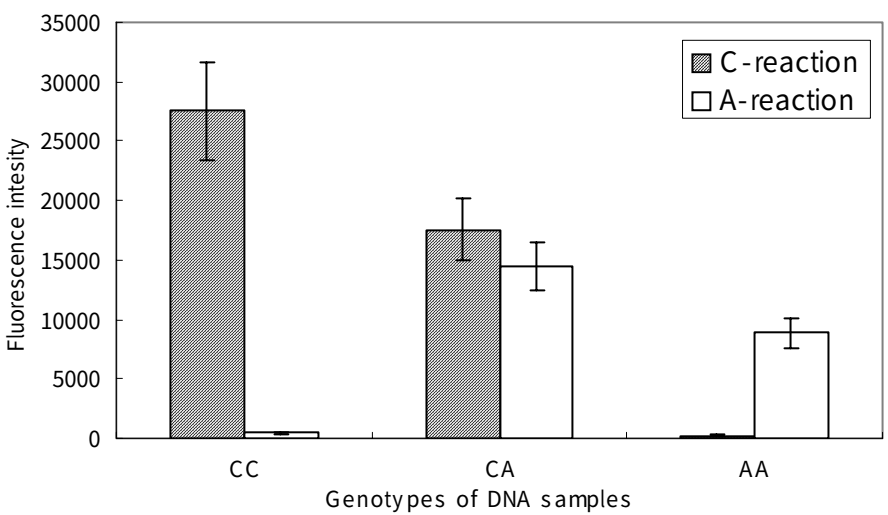
Name	SBE primer sequences (5′→3′)	Zip-code
SBE02-01	**CGATTACCCGCA**GCAGCACAACATCCCACAGC	1
SBE02-02	**CGATAGGTCGCA**TACCTGCAGCAGCACAACATC	2
SBE02-03	**AGGTCGCACGAT**CCGTGGCGTGTGGCG	3
SBE02-04	**CGCATACCCGAT**AGCAGCACAACATCCCACAG	4
SBE02-05	**CGCACGATGCTG**ACAGCGGGAGGTGGTCG	5
SBE02-06	**CGCAAGGTGCTG**CACTGGCCTCAACCAGTCC	6
SBE02-07	**AGGTCGATTACC**GACGCAGAAGCGGGCC	7
SBE02-08	**AGGTCGATGGTC**AACCAGTCCCACCTGTCCCAAC	8
SBE02-09	**CGATGCTGGGTC**TCCCATGAAGACGCAGAAGC	9
SBE02-10	**CGATGGTCAGGT**CCTACCTGCAGCAGCACAACA	10

The sequence complementary to the PNA zip-code sequence is bolded and underlined.

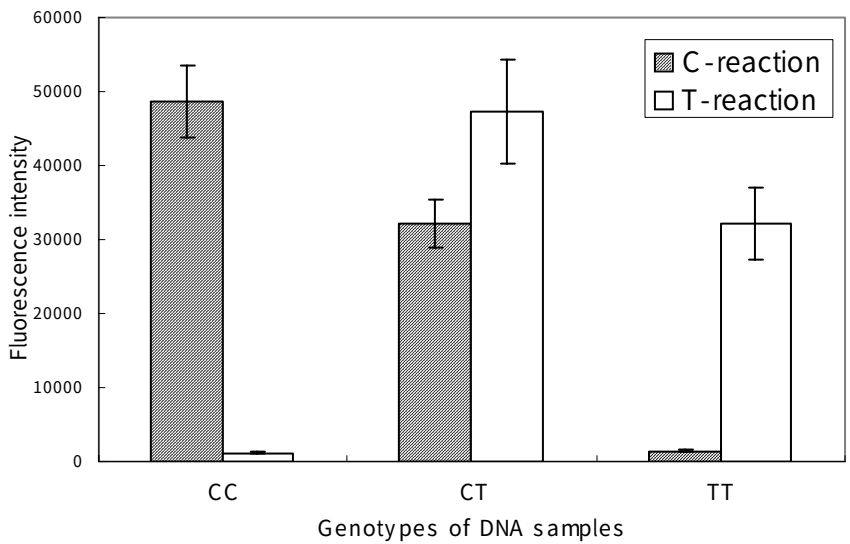
◆ Single point genetyping with a homozygote wild-type, homozygote mutant, & heterozygote mutant sample

- homozygote wild-type from an apparently healthy female
- MODY3 patient : two points of heterozygote mutant genotypes
  - CA at 1814 & CT at 1856
- Homozygote mutant: site-directed mutagenesis
- Homozygote samples: Signal to noise ratio > 20 for the two mutation positions
- Heterozygote sample: Ratio of the two signal intensity = 1.2 & 1.5

Mutation position 1814



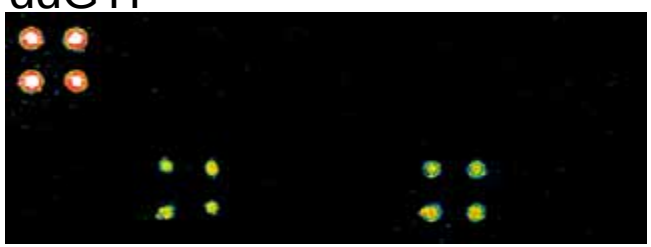
Mutation position 1856



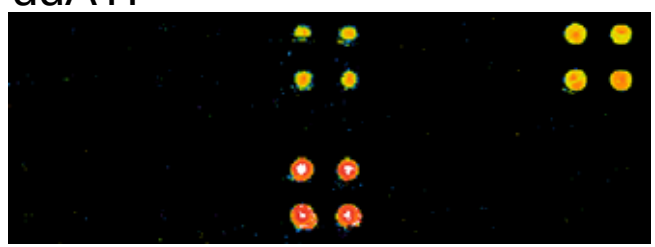
Diagnosis of HNF-1α mutations using multiplex SBE genotyping (Homozygote Wild-type)

Zip1 1821 (GG)	Zip2 1814 (CC)	Zip3 1788 (AA)	Zip4 1810 (CC)	Zip5 1833 (AA)
Zip6 1856 (CC)	Zip7 1910 (GG)	Zip8 1869 (AA)	Zip9 1905 (GG)	Zip10 1812 (TT)

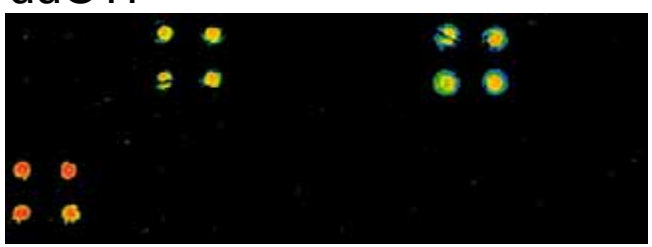
ddGTP



ddATP



ddCTP

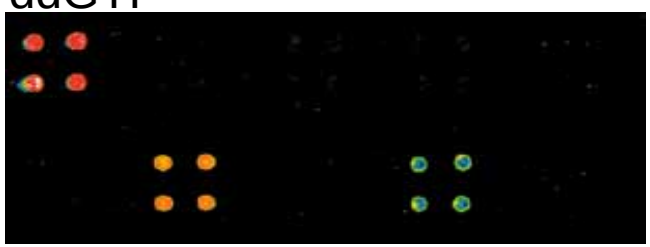


ddUTP

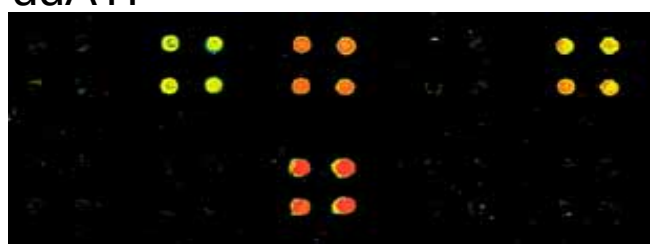
Diagnosis of HNF-1α mutations using multiplex SBE genotyping (Heterozygote mutant from a MODY patient)

Zip1 1821 (GG)	Zip2 1814 (CA)	Zip3 1788 (AA)	Zip4 1810 (CC)	Zip5 1833 (AA)
Zip6 1856 (CT)	Zip7 1910 (GG)	Zip8 1869 (AA)	Zip9 1905 (GG)	Zip10 1812 (TT)

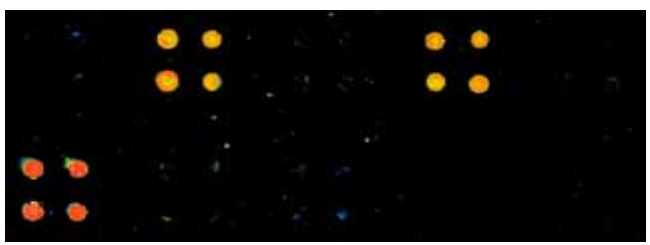
ddGTP



ddATP



ddCTP

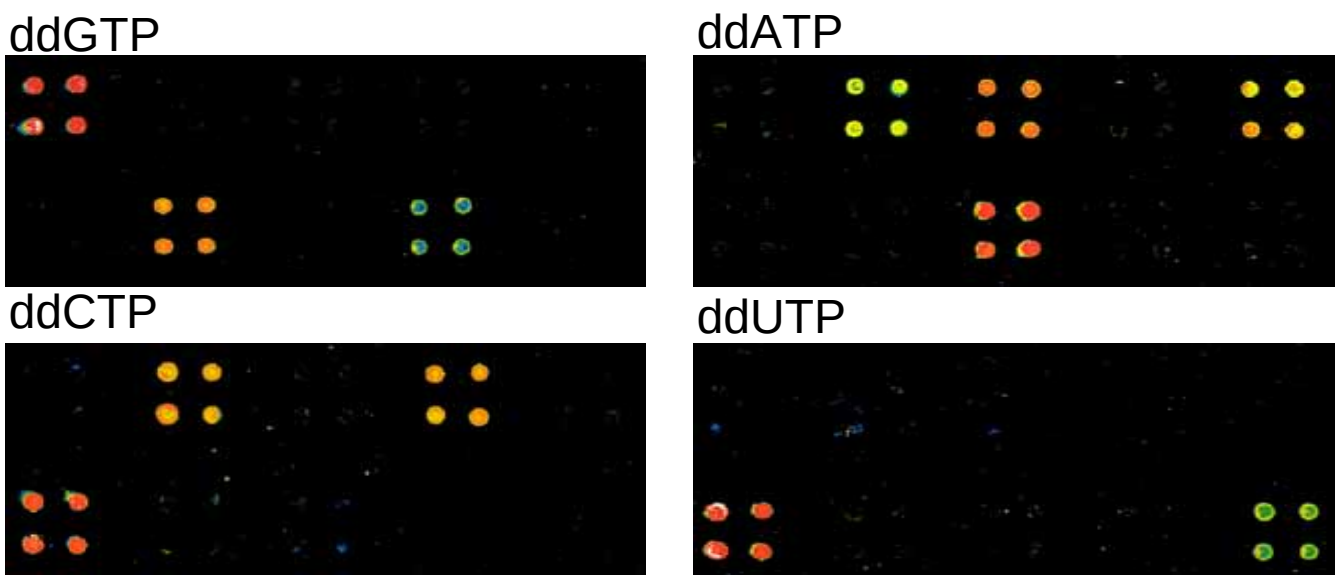


ddUTP

Diagnosis of HNF-1α mutations using multiplex SBE genotyping

(Homozygote mutant by site-directed mutagenesis)

Zip1 1821 (GG)	Zip2 1814 (AA)	Zip3 1788 (AA)	Zip4 1810 (CC)	Zip5 1833 (AA)
Zip6 1856 (CC)	Zip7 1910 (GG)	Zip8 1869 (AA)	Zip9 1905 (GG)	Zip10 1812 (TT)



Conclusions

• We developed expoxide-based immobilization strategy and achieved greatly improved binding capacity and reproducibility.

• We developed a SBE-based multiplex genotyping strategy utilizing PNA molecules as zip-code sequences and successfully achieved multiplex diagnoses of 10 HNF-1α mutations with a wild-type & mutant samples.

• This is the first serious report implementing the superior features of PNA molecules for the development of zip-code approaches and verifying the potential clinical utility of the strategy with real human samples

Park et al. *Nucleic Acids Research*, **33**(2), e19-e26, (2005)

Acknowledgement