

引物设计和Primer-BLAST的应用

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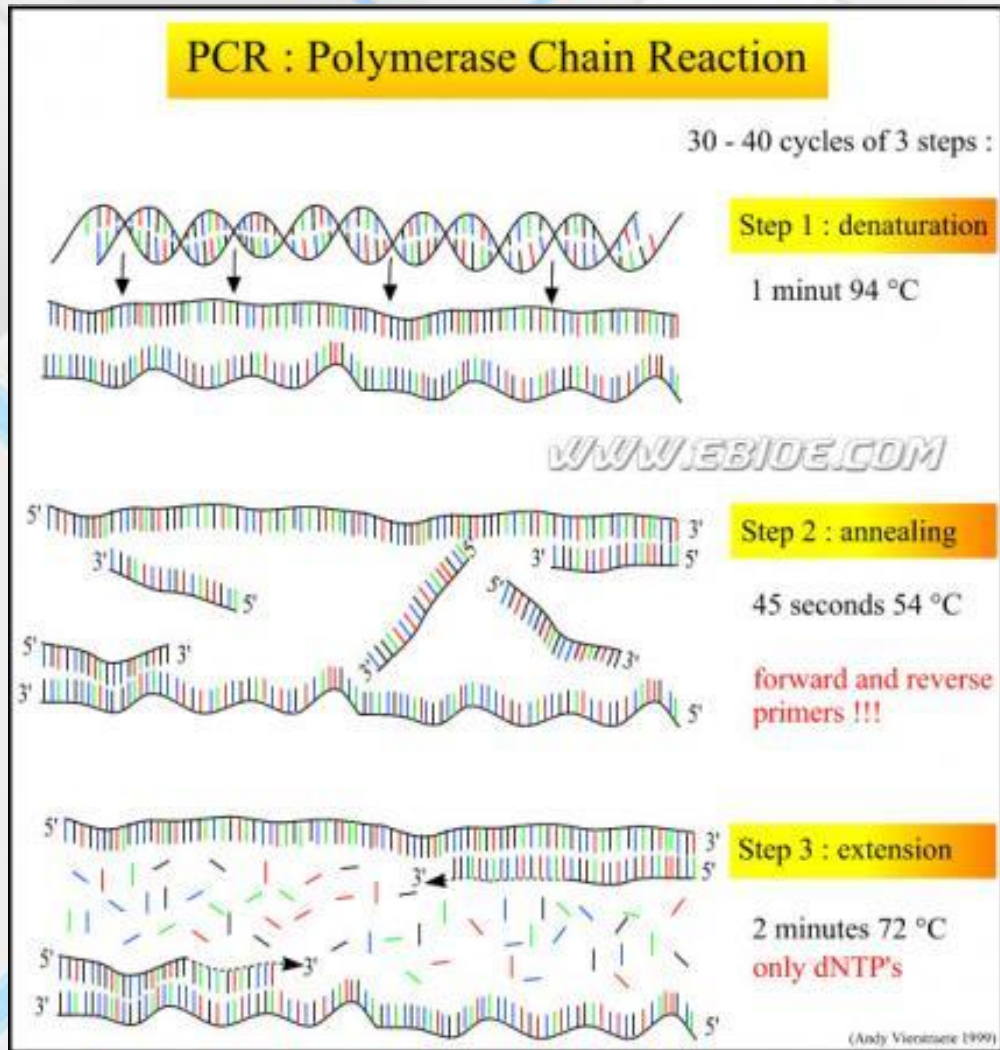
3.设计引物软件

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5.probeBase 简介

1.1 PCR (Polymerase Chain Reaction)

聚合酶链式反应



1971
Khorana
提出设想

1985
Kary Mullis
发明了PCR

1986年5月
Mullis在冷
泉港实验室
做专题报告

冷泉港实验室 (The Cold Spring Harbor Laboratory, 缩写 CSHL), 又译为科尔德斯普林实验室。

几不同的PCR技术

1. **扩增已知序列两侧DNA**的PCR：反向PCR（Inverse PCR, IPCR）、锚定PCR（anchored PCR）、RACE（Rapid Amplification of cDNA Ends）、连接介导的PCR（ligation-mediated PCR, LM-PCR）；
2. **检测有限量稀有靶序列**，即一对引物扩增产物不足以以通过凝胶电泳观察到的时：巢式PCR（nested PCR）；
3. **快速、灵敏、特异而准确定量**的PCR：实时荧光定量PCR（real-time quantitative PCR, RQ-PCR）。

引物

1.2 引物设计原则

引物特性及优化设计

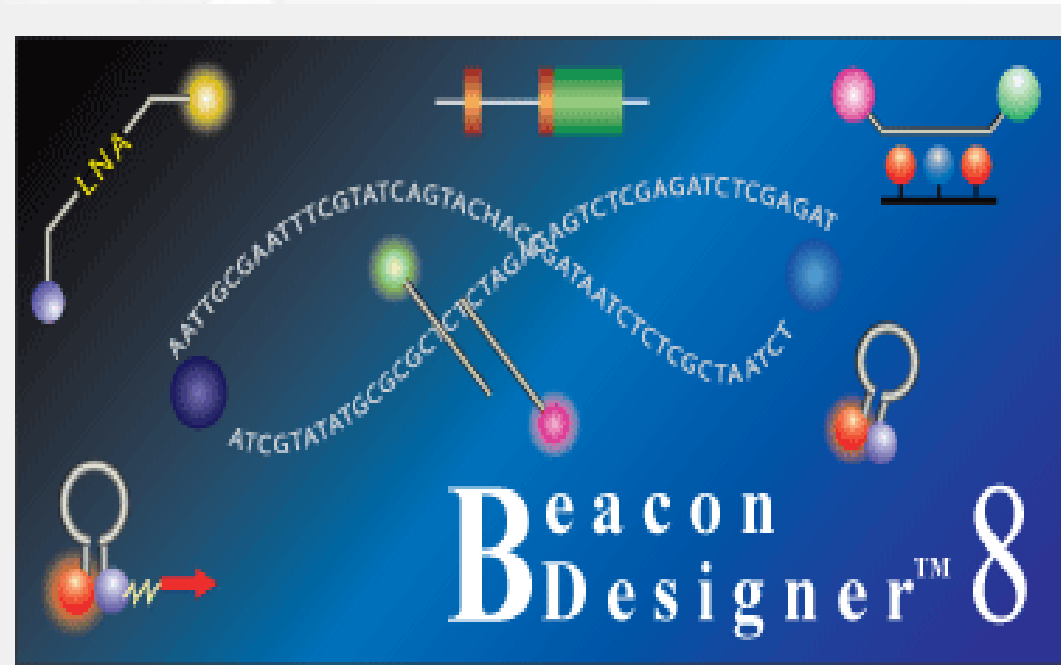
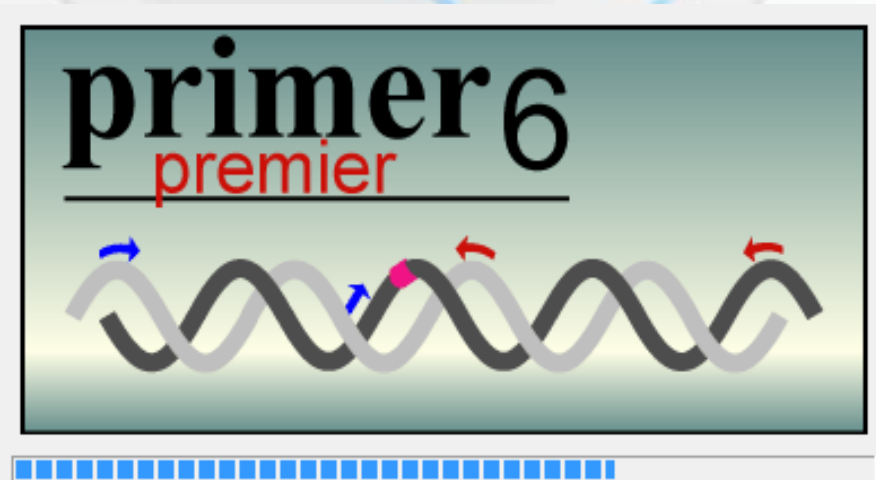
特性	优化
碱基组成	(G+C) 含量应在40%-60%，4种碱基要分布均匀；
长度	一般为18-27个核苷酸长度。上下游引物长度差别不能大于3bp；
重复和自身互补序列	不能有大于3bp的反向重复序列或自身互补序列存在；
上下游引物互补性	一个引物的3'末端序列不能结合到另一个引物的任何位点上；
解链温度 (T _m)	两个引物的T _m 值相差不能大于5°C，扩增产物与引物的T _m 值相差不能大于10°C
3'末端	引物3'末端碱基尽量为G或C，不能使3'末端有NNGC或NNCG序列
引物序列	不要有局部的GC rich或AT rich（特别是3'端），避开T/C或A/G的连续结构

决定引物退火温度（T_m值）最重要的因素就是引物的长度

$$T_m = (g + C) * 4 + (a + T) * 2$$

1.3常用引物设计软件

Primer Premier 6 (P6)、**Beacon Designer 8 (DB)** (自动搜索)



Oligo 6 (引物评价)

Vector NTI Suit (综合分析)

Primer Express (实时定量PCR引物和探针设计)

*Omiga

*Dnastar

1.3.1 Primer 6和BD 8设计引物参数

- 斑马鱼基因serpine1 : 抑制血管生成和新陈代谢
- NCBI Reference Sequence: NM_001114559.1

Primer Premier 6(PP6) 参数

Tm: 57°C±2°C Length: 18± 27 bp

Amplicon Length: 80—200 bp (G+C)%=40%—60%



Beacon Designer 8(BD8)参数

Tm: 66°C±2°C Length: 18± 27 bp

Amplicon Length: 80—200 bp (G+C)%=40%—60%

1.3.2 Beacon Designer 8 (BD主界面)

The screenshot shows the main interface of Beacon Designer 8.14. The window title is "Beacon Designer 8.14 - D:\APP\Beacon Designer 8.14\BDProjects\2015.11.18.bdp". The menu bar includes File, Edit, View, Analyze, Tools, Online, Assays, and Help. The toolbar contains various icons for file operations and analysis. A dropdown menu is open, showing design structure options: SYBR[®] Green Design, Beacon Design, FRET Design, HRMA Design, MethyLight TaqMan[®] Design, Scorpions[®] Design, SYBR[®] Green Design, and TaqMan[®] Design. The main workspace is divided into several sections: "Sequence Information" with a table for sequence details, "Primer Properties" with a table for primer characteristics, and "BLAST Information", "SNP Information", and "EXON Exon Information" tabs. Two buttons, "All Primers..." and "All Structures", are circled in red. The status bar at the bottom shows "Ready".

功能选择区

设计结构选项

序列基本信息

#	Definition	Length
---	------------	--------

序列碱基详细信息窗口

其他信息

引物特征

Accession Number/Name	Status	Rating	Sequence	Position	Length bp	T _m °C	GC %	Hairpin ΔG kcal/mol	Self Dimer ΔG kcal/mol	Run Length bp	GC Clamp	T _a Opt °C	Cross Dimer ΔG kcal/mol
-----------------------	--------	--------	----------	----------	--------------	----------------------	------	------------------------	---------------------------	------------------	----------	--------------------------	----------------------------

所有引物和结构选择

引物显示区

1.3.3 BD 8操作流程

(1) 序列导入

(2) 参数选择

(3) 结果分析

(4) 引物检验

New Sequence

Sequence Definition: Serpine1 **序列名称**

Sequence

mRNA碱基序列

TGGCAGGCACACAGAGCAGCTCTAGGCTTCCACTGCATTGGATGATCACAGTAACGCCTTACATCTACAG
GAAACTCTACAGAAGTAAAGCATCTTTAAGATATTAGAATGCAGAGCCTGAGTGTGCTACTGATCTTTG
CCTTTGCGCATCAAGCTTGTGCAACCTTATCCAGGACAAAACAGACAGATTTTGGGCTACAGGTGTTTGCT
AAGCCGTCAGTCTGCTCCGGATCGAAATCTGGCTCTCTCCATATGGTATCGCCTCGGTGCTGGGAAT
GCTCAGATGGGTGCCTATGGCGCTACTCTAAAACCTGCTCGCCTCCAAGATGGGCTACTCTCTGCAAGAAA
AGGAATGCCAAACTGCAGAGCCTTCTTCAGAGCATCTGGCCAGTGAAGATGGAGTGGAGGTAGCCAGC
GGTCATGGTTGACCGGAAGATCTGAGCAAAGCCTTCCAGAG
GTCCACACCAGATAGACTTCAAACTCCTGGACGTCTGACC
CACTGATGGAATGATTTCTGAGTTCCTCCCATCTGGAGTGTGAGTGAACGACACGGTTAGTCTTCCTG
ATGCCCTGCACTTCCACGGGGTCTGGAAGACACCATTTGACCCAGAAACACTCGAGAGCAGCTCTTCCA
ACAGTTAACGGCAGTGTGTATCTGTTCCCATGATGACAACCACTCAAAAATCAACTATGGTGAATTCG
GCTAAGGATGGCGTGGACTATGACGTCATCGAGATGCCTTATGAGGGGGAGTCGATAAGTATGCTCCTG
TGACACCTTTTGAGAAGGACGTGCCTGTCTGCCTTGAACAAAGAGCTGAGCAGCAGCAGGATCCACCA
TGGAGACAAGAAATGAGGAAGATAAGCAAAACAGCTGTCTATTCCAAGTCTCCATGGATACTGAAATTG
TCTGAAGTCCCACTGAGCAGAATGGGTCTTGGAGACATTTTCAGCCAGAGTAGAGCTGATTTCTCTCGC
TTACTACTGAGGAACCTTTATGTGTATCCAAGGTCCTTCAGAGAGTAAAACCTTGAAGTGAATGAAGAAGG

Add Cancel Help

Sequence Information | Search Status

#	Definition	Length
*000001	Serpine1	1789

序列长度 1789

```
1 TGGCAGGCAC ACAGAGCAGC TCTAGGCTTC CACTGCAATG GATGATCACA GTAADGCCTT
61 ACATCTACAG GAAACTCTA CAGAAGTAAA GCATCTTTAA GATATTCAGA ATCCAGAGCC
121 TGAGTGTGCT ACTGATCTTT GCCCTTTGGG CATCAAGCTT GTGCAACCTT ATCCAGGACA
181 AACAGACAGA TTTTGGGCTA CAGGTGTTTG CTGAAGCCGT CCAGTCTGCT CCGGATCGAA
241 ATCTGGCTCT CTCTCCATAT GGTATGCGCT CGGTGCTGGG AATGGCTCAG ATGGGTGCTT
301 ATGGCGCTAC TCTAAAAC TGCGCTCCA AGATGGGCTA CTCTCTGCAA GAAAGAGGAA
361 TGCCAAACT GCAGAGGCTT CTTGAGAGAG ATCTGGCCAG TGAAGATGGA GTGGAGGTAG
421 CCAGCGGGGT CATGGTTGAC CGGAAGATCA TCTTGAAAA GGTCTTTAGG CCGACTTGA
481 GCAAAGCCTT CCAGAGGCTC CCACACCAGA TAGACTTCAG CCAGCCAGAG ATGGCCAGGC
541 AGTGATCAA CTCTGGAGC TCTGACCACA CTGATGGAAT GATTTCTGAG TTCTCTCCAT
601 CTGGAGTCTT GAGTGAAGT ACACGGTTAG TCTTCTGAA TGCCCTGCAC TTCCACGGGG
661 TCTGGAAGC ACCAATTGAC CCCAGAAACA CTCGAGACCA GCTCTCCAC ACAGTTAAGC
721 GCAGTGTGCT ATCTGTCCC ATGATGACAA CCACTCAAAA ATTCAACTAT GGTGAGTTG
781 TGTCTAAGGA TGCCGTGGAC TATGACGTCA TCGAGATGCC TTATAGGGGG GAGTGTGATA
841 GTATGCTCTT GGTGACACT TTTGAGAAAG ACCTGCCCTT GTCTGCCCTT AACAAAGAC
```

1.3.3 BD 8操作流程

(1) 序列导入

(2) 参数选择

(3) 结果分析

(4) 引物检验

Primer Search

Parameter Type

Default Last Searched User Defined: Select a...

Restore

Search Parameters | Primer Parameters

Primer parameters

Tm: 57.0 +/- 5.0 °C

Ta: 55.0 +/- 5.0 °C

Length: 18 To: 27 bp

Primer pair parameters

Amplicon Length: 80 To: 200 bp

Alternate Primer Pairs: 5

Save as User Defined... Advanced...

Search Cancel Help

基本参数

Select a...

B

short

short1

qPCR

>200bp

<100bp

RT-PCR1

ddPCR

Advanced Search Parameters

Advanced primer parameters

Hairpin Maximum ΔG (3' End): 3.0 -kcal/mol

Hairpin Maximum ΔG (Internal): 5.0 -kcal/mol

3' End Maximum ΔG : 12.0 -kcal/mol

Self Dimer Maximum ΔG (3' End): 5.0 -kcal/mol

Self Dimer Maximum ΔG (Internal): 6.0 -kcal/mol

Run/Repeat (dinucleotide) Maximum Length: 4 bp

G/C clamp - Consecutive G/C's at 3' End: 1

GC% 40.0 To: 60.0

Advanced primer pair parameters

Maximum Ambiguous Bases in Amplicon: 0

Maximum Primer Pair Tm Mismatch: 4.0 °C

Cross Dimer Maximum ΔG (3' End): 5.0 -kcal/mol

Cross Dimer Maximum ΔG (Internal): 6.0 -kcal/mol

OK Cancel Help

高级参数

ΔG 值: DNA双链形成时所需的自由能, 反映了双链结构内部碱基对的相对稳定性。

应选择3'端 ΔG 值较低(绝对值不超过9), 而5'端和中间 ΔG 值过高, 容易在错配位点形成双链结构并引发DNA聚合反应。

(能值越高越容易结合)

1.3.3 BD 8操作流程

(1) 序列导入

(2) 参数选择

(3) 结果分析

(4) 引物检验

The screenshot shows the SYBR Green Design software interface. The top toolbar includes icons for file operations, sequence analysis, and design parameters. The main window is divided into several panes:

- Sequence Information:** Shows a search status for accession number *000001 with a 'Best' result.
- Sequence View:** Displays a DNA sequence with a primer pair highlighted in blue and red. The primer sequences are CAGTGAAGATGGAGTGGAGGTAG (sense) and TCCTGGCTGGCTGAAGTC (anti-sense).
- Primer Properties:** A table providing detailed metrics for the sense, anti-sense, and product sequences.

The 'EXON Exon Information' tab is highlighted with a red circle and a red triangle with the number '2', indicating the current step in the workflow. Below this tab, the text '外显子信息' (Exon Information) is written in red.

	Rating	Sequence	Position	Length bp	Tm °C	GC %	Hairpin ΔG kcal/mol	Self Dimer ΔG kcal/mol	Run Length bp	GC Clamp	TaOpt °C	Cross Dimer ΔG kcal/mol
Sense	91.2	CAGTGAAGATGGAGTGGAGGTAG	398	23	65.9	52.2	0.0	0.0	2	2		
AntiSense	90.7	TCCTGGCTGGCTGAAGTC	531	19	65.6	57.9	0.0	0.0	2	1		
Product	92			134	82.2						62.3	-1.2

Below the table, the text '得分' (Score) is written in red, and '长度 Tm值 GC%' (Length Tm value GC%) is written in red, indicating the key parameters for primer evaluation.

1.3.3 BD 8操作流程

(1) 序列导入

(2) 参数选择

(3) 结果分析

(4) 引物检验

BD All Primers 所有引物

Sort for: Sense Primer Anti-sense Primer Product

#	Rating	Sequence	Position	Length bp	Tm °C	GC %	Hairpin ΔG kcal/mol	Self Dimer ΔG kcal/mol	Cross Dimer ΔG kcal/mol
1.	91.2	CAGTG...	398	23	65.9	52.2	0.0	0.0	-1.2
	90.7	TCTCT...	531	19	65.6	57.9	0.0	0.0	
	92.0			134	82.2				
2.	90.9	CCGTA...	1175	19	65.7	57.9	0.0	0.0	-0.6
	89.6	GTTCA...	1332	27	66.1	40.7	-0.6	-0.6	
3.	91.8			158	80.9				-0.6
	90.9	CCGTA...	1175	19	65.7	57.9	0.0	0.0	
4.	88.8	GGTTC...	1333	26	65.9	42.3	-0.6	-0.6	-0.6
	91.6			159	81.0				
5.	91.2	CAGTG...	398	23	65.9	52.2	0.0	0.0	-1.2
	90.8	CTCTG...	530	22	66.4	54.5	0.0	0.0	
6.	91.6			133	82.3				-1.2
	87.4	CACGG...	622	22	65.4	50.0	0.0	-0.6	
7.	90.6	CATAG...	804	22	65.6	50.0	0.0	0.0	-0.7
	91.1			183	81.6				
8.	91.2	CAGTG...	398	23	65.9	52.2	0.0	0.0	-1.2
	89.9	TCTCT...	531	21	66.7	52.4	0.0	0.0	
9.	90.8			134	82.2				-1.2

Buttons: All Structures, Replace, Cancel, Help

引物得分降序

FP: AGTGAAGATGGAGTGGAGGTAG

RP: TCTGGCTGGCTGAAGTCTATC

BD Primer Secondary Structures 引物二级结构

Accession Number/Name: *000001

Self Dimer | Hairpin | Cross Dimer | Runs | Repeats

#	Structure	ΔG ...
1.	5' CAGTGAAGATGGAGTGGAGGTAG 3' 3' CTGAAGTCGGTCGGTCTCT 5'	-1.2
2.	5' CAGTGAAGATGGAGTGGAGGTAG 3' 3' CTGAAGTCGGTCGGTCTCT 5'	-1.2
3.	5' CAGTGAAGATGGAGTGGAGGTA... 3' CTGAAGTCGGTCGGTCTCT 5'	-1.2
4.	5' CAGTGAAGATGGAGTGGAGGTAG 3' 3' CTGAAGTCGGTCGGTCTCT 5'	-1.0
5.	5' CAGTGAAGATGGAGTGGAGGTAG 3' 3' CTGAAGTCGGTCGGTCTCT 5'	-1.0
6.	5' CAGTGAAGATGGAGTGGAGGTAG 3' 3' CTGAAGTCGGTCGGTCTCT 5'	-0.7
7.	5' CAGTGAAGATGGAGTGGAGGTAG 3' 3' CTGAAGTCGGTCGGTCTCT 5'	-0.7

Buttons: OK, Help

同向引物二聚体

发夹结构

交叉二聚体

连续序列

“-AA-”

重复序列

“-GCCG-”

1.4常用在线设计引物工具

- <http://www.idtdna.com/site> (Integrated DNA Technologies)
- Web Primer: <http://www.yeastgenome.org/cgi-bin/web-primer> (酵母基因组数据库提供)
- Primer Design: http://bioweb.uwlax.edu/GenWeb/Molecular/seq_anal/primer_design/primer_design.htm (较基础全面)

- ★ **NCBI Primer-Blast**

<http://www.ncbi.nlm.nih.gov/tools/primer-blast/> (引物设计、验证)

Primer Design

Current Level

- Seq Editor
- Searches
- Seq. Comparison
- Alignment
- Restriction Map
- Translation & ORF
- Reverse Comp
- Primer Design**
- Splice Sites
- Genomics
- Protein Motifs
- Protein structure
- Transmembrane

What is a primer?

A primer is a short synthetic oligonucleotide which is used in many molecular techniques from [PCR](#) to [DNA sequencing](#). These primers are designed to have a sequence which is the reverse complement of a region of template or target DNA to which we wish the primer to anneal.

Diagram illustrating primer annealing:

```
3'          5'
TGACCTGAAAAGAC ← Primer

GATGGACTGATTACCGATGACTGGACTTTTCTG ← Template
5'          3'

↓

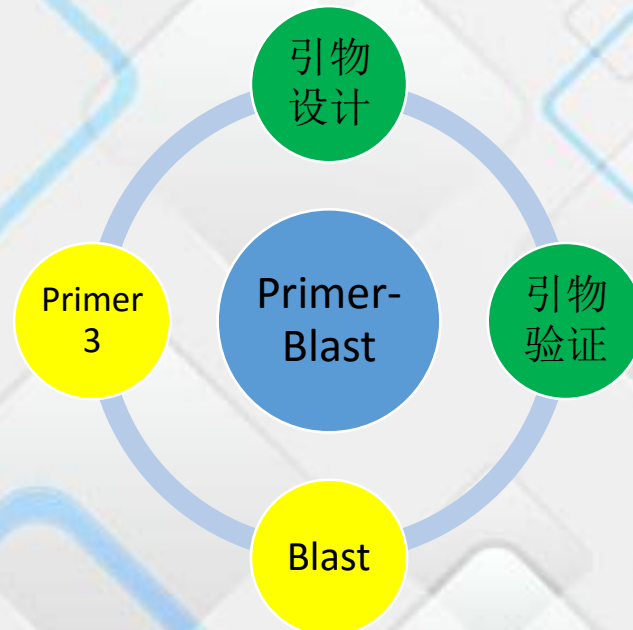
3'          5'
TGACCTGAAAAGAC ← Annealing
GATGGACTGATTACCGATGACTGGACTTTTCTG
5'          3'
```

1.4.1 NCBI Primer-Blast简介



Primer-BLAST，能在线**设计**引物，并**验证**设计好的引物。整合了目前流行的**Primer3**软件，同时进行**NCBI的Blast**进行引物特异性验证。**Primer-BLAST**免除了用另一个站点或工具设计引物的步骤，设计好的引物直接用**Blast**进行引物特异性验证。

并且，**Primer-BLAST**能设计出只扩增某一特定剪接变异体基因的引物——这是用来衡量基因在特定组织中特异性表达的重要特征。



1.4.2 NCBI Primer-Blast引物设计



(1) 导入模版链序列

NCBI/ Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST).

Reset page Save search parameters Retrieve recent results Publication Tips for finding specific primers

PCR Template

Enter accession, gi, or FASTA sequence (A refseq record is preferred) Clear

```
>gi|167621479|ref|NM_001114559.1| Danio rerio serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (serpin1), mRNA
GATGGCCGGGGACACAGAGCAGCTCTAGGCTTCCACTGCATTGGATGATCACAGTAACGCCTTACATCT
ACAGGGAAACTCTACAGAAGT 模板链序列或登录号 AGAGCCTGAGTGTGCTACTGA
TCTTTGCCCTTTGCGCATCAA.....AGACAGATTTTGGGCTACAGG
```

Or, upload FASTA file 浏览... 未选择文件.

Range

Forward primer From To Clear

Reverse primer

选择上下游引物的范围

热力学参数表
盐度修正公式

(2) 引物基本参数

Primer Parameters

Use my own forward primer (5'->3' on plus strand) Clear

Use my own reverse primer (5'->3' on minus strand) Clear

PCR product size Min Max

of primers to return

Primer melting temperatures (T_m) Min Opt Max Max T_m difference

已设计引物

PCR产物大小

引物T_m值

The **T_m calculation** is controlled by **Table of thermodynamic parameters** and **Salt correction formula** (under advanced parameters). The default Table of thermodynamic parameters is "SantaLucia 1998" and the default Salt correction formula is "SantaLucia 1998" as recommended by primer3 program.

(3) 外显子/内含子选项

Exon/intron selection A refseq mRNA sequence as PCR template input is required for options in the section

Exon junction span No preference 外显子连接跨度

Exon junction match Exon at 5' side: 7 Exon at 3' side: 4
Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction

Intron inclusion Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range Min: 1000 Max: 1000000

(4) 引物特异性检验参数

Primer Pair Specificity Checking Parameters

Specificity check Enable search for primer pairs specific to the intended PCR template

Search mode Automatic

Database Refseq RNA (refseq_rna) 检索数据库选择

Exclusion Exclude predicted Refseq transcripts (accession with XM, XR prefix) Exclude uncultured/environmental sample sequences

Organism 物种限定 Danio rerio (taxid:7955)
Enter an organism name (or organism group name such as enterobacteriaceae, rodents), taxonomy id or select from the suggestion list as you type.

[Add more organisms](#) 增加物种

Entrez query (optional)

Primer specificity stringency Primer must have at least 2 total mismatches to unintended targets, including at least 2 mismatches within the last 5 bps at the 3' end. Ignore targets that have 6 or more mismatches to the primer.

Max target size 4000

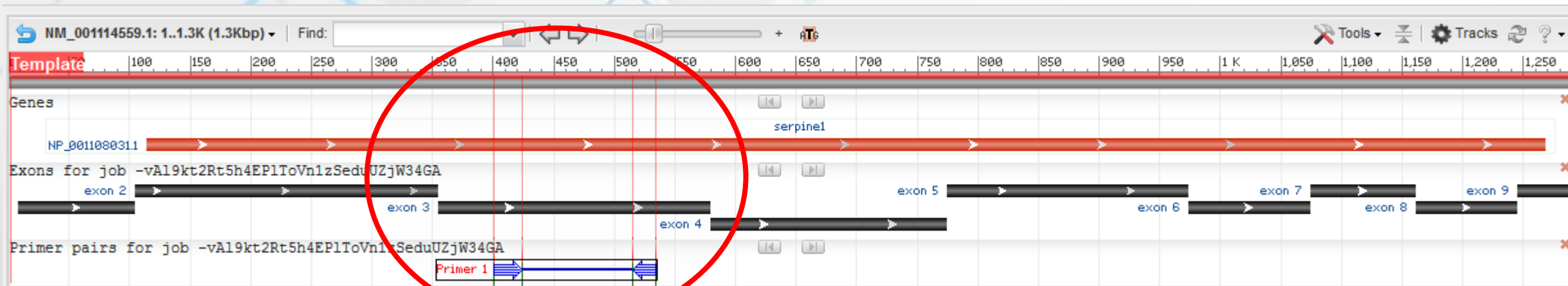
Splice variant handling Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)

(5) 高级选项中引物参数

	Min	Opt	Max	
PCR Product Tm	<input type="text"/>	<input type="text"/>	<input type="text"/>	PCR产物Tm值
Primer Size	<input type="text" value="15"/>	<input type="text" value="20"/>	<input type="text" value="25"/>	引物长度
Primer GC content (%)	<input type="text" value="20.0"/>	<input type="text" value="80.0"/>		引物GC含量
GC clamp	<input type="text" value="0"/>			
Max Poly-X	<input type="text" value="5"/>			最大单核苷酸聚合体
Max 3' Stability	<input type="text" value="9"/>			最大3'端稳定性
Max GC in primer 3' end	<input type="text" value="5"/>			最大3'端GC含量
Secondary Structure Alignment Methods	<input type="checkbox"/> Use Thermodynamic Oligo Alignment <input type="checkbox"/> Use Thermodynamic Template Alignment (warning: search may be very slow with this option on)			

GC clamp: GC夹，在一侧引物的5'端加上一个30-40bp的GC结构，这样在PCR产物的一侧可产生一个高熔点区，使相应的感兴趣的序列处于低熔点区而便于分析。能极大提高突变检出率。

(6) 结果



Primer pair 1

	Sequence (5'→3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAGTGAAGATGGAGTGGAGGTAG	Plus	23	401	423	59.87	52.17	3.00	0.00
Reverse primer	TCTCTGGCTGGCTGAAGTC	Minus	19	534	516	59.02	57.89	3.00	2.00
Product length	134								

Products on intended target

>[NM_001114559.1](#) Danio rerio serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (serpine1), mRNA

product length = 134

```
Forward primer 1 CAGTGAAGATGGAGTGGAGGTAG 23
Template 401 ..... 423

Reverse primer 1 TCTCTGGCTGGCTGAAGTC 19
Template 534 ..... 516
```

1.4.3 NCBI Primer-Blast引物验证



如果你已经设计好了引物，要验证引物的好坏。可以在Primer-Blast中进行，在Primer Parameters区填入你的一对引物。选择好验证的目标数据库（在specificity check区选择）。根据需要可设置产物的大小，T_m值等。

(1) 输入已知引物

Primer Parameters

Use my own forward primer
(5'→3' on plus strand) [Clear](#)

Use my own reverse primer
(5'→3' on minus strand) [Clear](#)

PCR product size

Min Max

(2) 调整参数

特异性参数选择同前面引物设计

Primer Pair Specificity Checking Parameters

Specificity check	<input checked="" type="checkbox"/> Enable search for primer pairs specific to the intended PCR template ?
Search mode	Automatic ?
Database	Refseq RNA (refseq_rna) ?
Exclusion	<input checked="" type="checkbox"/> Exclude predicted Refseq transcripts (accession with XM, XR prefix) <input checked="" type="checkbox"/> Exclude uncultured/environmental sample sequences ?
Organism	Danio rerio (taxid:7955) ? Enter an organism name (or organism group name such as enterobacteriaceae, rodents), taxonomy id or select from the suggestion list as you type. ? Add more organisms
Entrez query (optional)	<input type="text"/> ?
Primer specificity stringency	Primer must have at least <input type="text" value="2"/> total mismatches to unintended targets, including at least <input type="text" value="2"/> mismatches within the last <input type="text" value="5"/> bps at the 3' end. ? Ignore targets that have <input type="text" value="6"/> or more mismatches to the primer. ?
Max target size	<input type="text" value="4000"/> ?
Splice variant handling	<input checked="" type="checkbox"/> Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input) ?

(3) 结果

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGTGAAGATGGAGTGGAGGTAG	22	58.63	50.00	2.00	0.00
Reverse primer	TCTGGCTGGCTGAAGTCTATC	21	59.24	52.38	3.00	3.00

Products on target templates

>[NM_001114559.1](#) Danio rerio serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (serpine1), mRNA

product length - 131
Forward primer 1 AGTGAAGATGGAGTGGAGGTAG 22
Template 402 423
Reverse primer 1 TCTGGCTGGCTGAAGTCTATC 21
Template 532 512

1.5 Primer-Blast小结

(1) Primer-Blast设计的引物

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAGTGAAGATGGAGTGGAGGTAG	23	59.87	52.17	3.00	0.00
Reverse primer	TCTCTGGCTGGCTGAAGTC	19	59.02	57.89	3.00	2.00

软件设计的引物，经Primer-Blast验证

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGTGAAGATGGAGTGGAGGTAG	22	58.63	50.00	2.00	0.00
Reverse primer	TCTGGCTGGCTGAAGTCTATC	21	59.24	52.38	3.00	3.00

- (2) 数据库是经过专家注释的数据，这样可以给出更准确的结果。特别是Tm值、GC含量计算，引物二级结构、引物互补预测；
- (3) 尽量使用没有冗余的数据库（如refseq_rna 或 genome database），nr数据库包括了太多的冗余的序列，会干扰引物的设计。

1.6 probeBase 2016简介

<http://probebase.csb.univie.ac.at/node/8>

简介: **probeBase** is a curated database of **rRNA-targeted** oligonucleotide **probes** and **primers**.
probeBase 是一个针对设计rRNA的寡聚核苷酸探针和引物的精选数据库。
主要用于设计rRNA探针和引物。



The screenshot shows the homepage of the probeBase 2016 website. The header features the DOME logo (Division of Microbial Ecology) and the text "probeBase 2016" with the tagline "An online resource for rRNA-targeted oligonucleotides". Below the header is a navigation menu with buttons for Home, Search, Match, Proxy, Lists, Submission, Links, and Credits. The main content area displays "Welcome to probeBase" and a brief description: "probeBase is a curated database of rRNA-targeted oligonucleotide probes and primers."

1.6 probeBase 2016功能

Search

Search probeBase for target organisms, probe names, primers, target sites, references, etc.

Match

Match rRNA sequence(s) against probeBase and find all published probes targeting your sequence(s).

Proxy

Match partial rRNA sequence(s) against full-length sequences in SILVA and find published probes potentially targeting your sequence(s). <http://www.arb-silva.de>

高质量rRNA数据库

Lists

View lists of probes (according to probe categories), primers, microarrays, references, etc.

Submission

Submit new or missing probes to probeBase.

Links

Websites relevant to ribosomal RNA



搜索探针和引物



匹配rRNA序列找探针



匹配部分rRNA序列



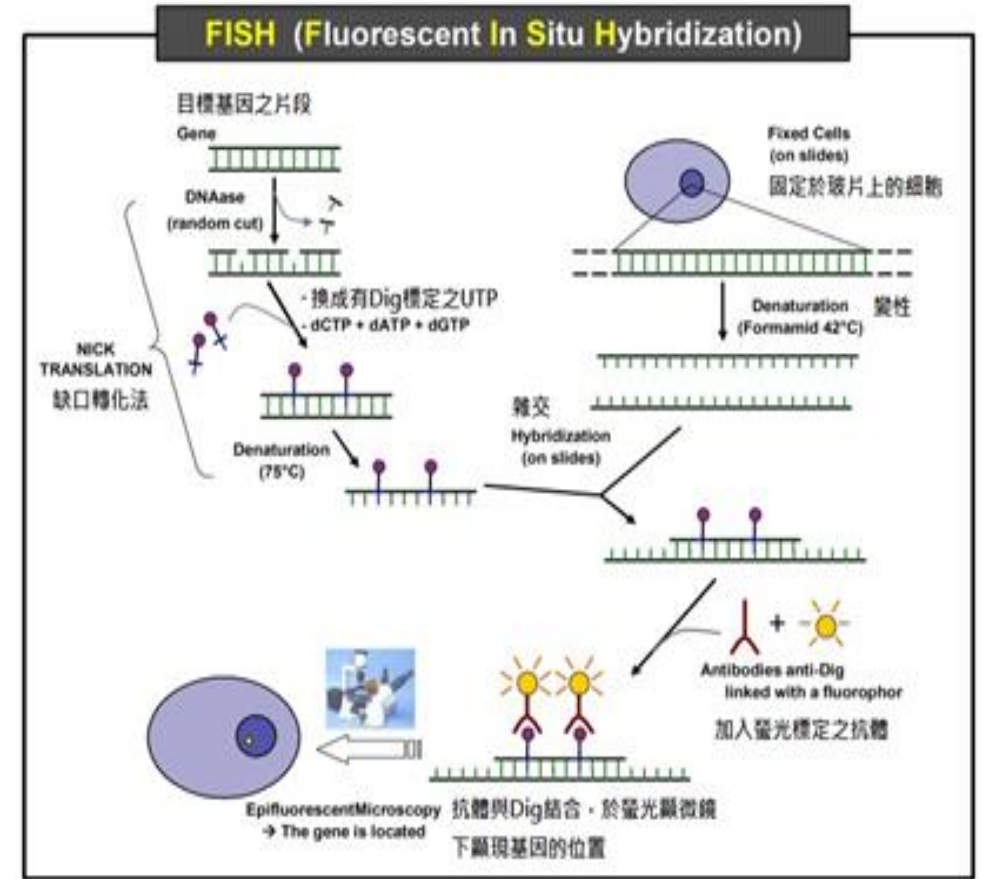
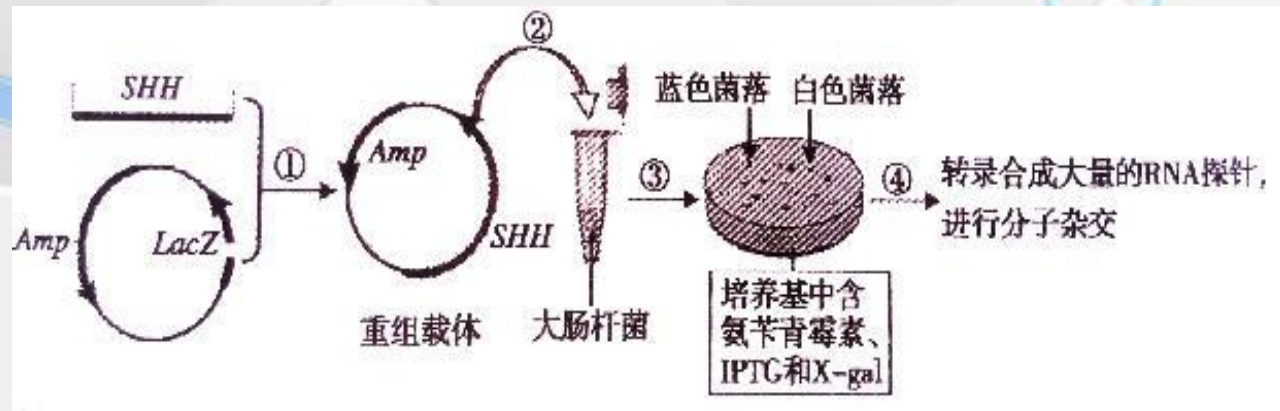
参考列表



提交

1.6 probeBase应用实例

(1) 菌种鉴定：收集已培养或未培养微生物的16S rRNA序列信息，常用于鉴定新分离的菌株。probeBase 可用来检索与16S rRNA 有关的探针。



圖一、FISH 機制示意圖

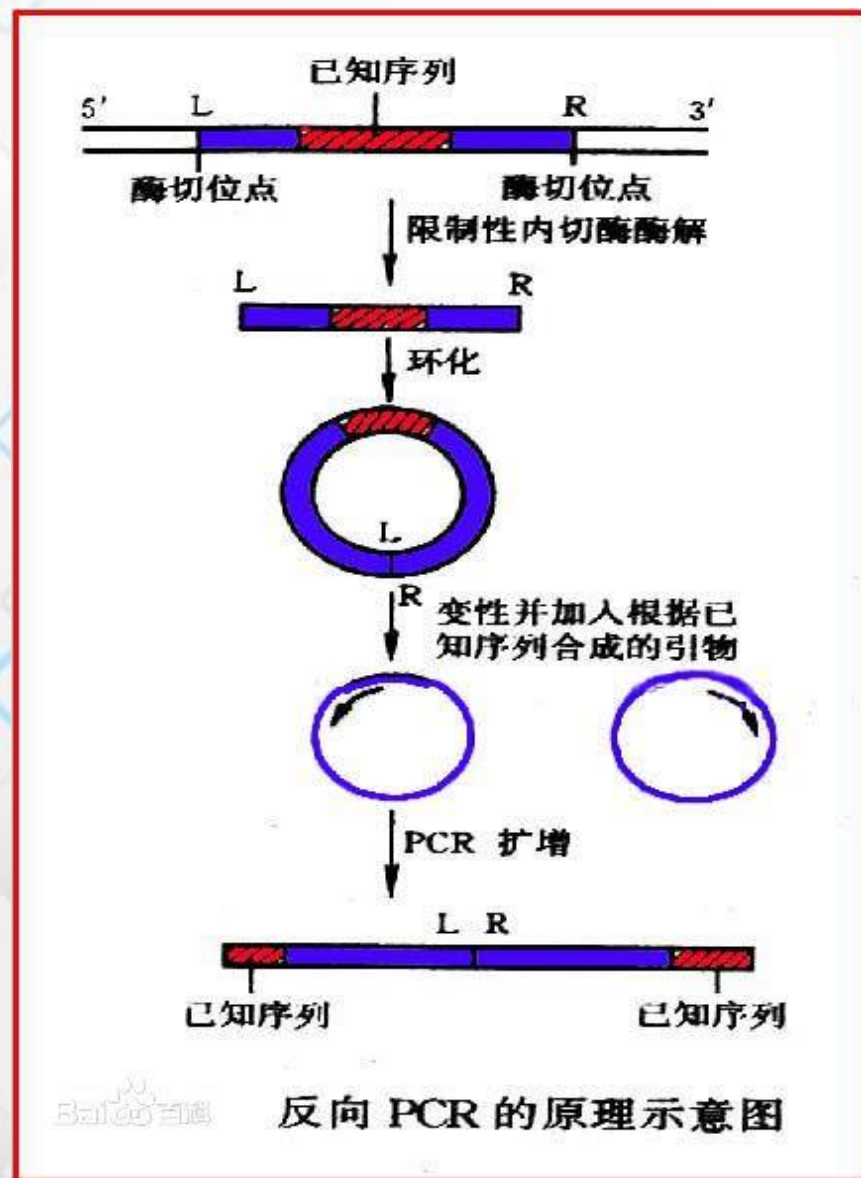
(2) 原位杂交：FISH 技术成功应用的关键在于设计和获得具有高灵敏性和专一性的寡核苷酸探针以减少干扰，可使用ARB 软件包结合probeBase 数据库等多种方法进行探针分析和设计。

1.7 参考文献

1. Alexander Loy, Frank Maixner, Michael Wagner and Matthias Horn* (2006) **probeBase—an online resource for rRNA-targeted oligonucleotide probes: new features 2007**, *Nucleic Acids Research*, Vol. 35, D800–D804 Database issue
2. Loy,A., Horn,M. and Wagner,M. (2003) **probeBase: an online resource for rRNA-targeted oligonucleotide probes**. *Nucleic Acids Res.*,31, 514–516.
3. Jian Ye1*, George Coulouris1 , Irena Zaretskaya1 , Ioana Cutcutache2 , Steve Rozen2 and Thomas L Madden1. **Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction**, Ye et al. *BMC Bioinformatics* 2012, 13:134
- 4.刘驰, 李家宝, 芮俊鹏, 安家兴, 李香真.**16S rRNA 基因在微生物生态学中的应用**.*生态学报*, 2015, 35(9):2769-2788

附1：反向PCR（Inverse PCR, IPCR）

反向PCR可用于研究与已知DNA区段相因此又可称为染色体缓移或染色体步移。这时选择的引物虽然与核心DNA区两末端序列互补，但两引物3'端是相互反向的。扩增前先用限制性内切酶切样品DNA，然后用DNA连接酶连接成一个环状DNA分子，通过反向PCR扩增引物的上游片段和连接的未知染色体序列，下游片段。

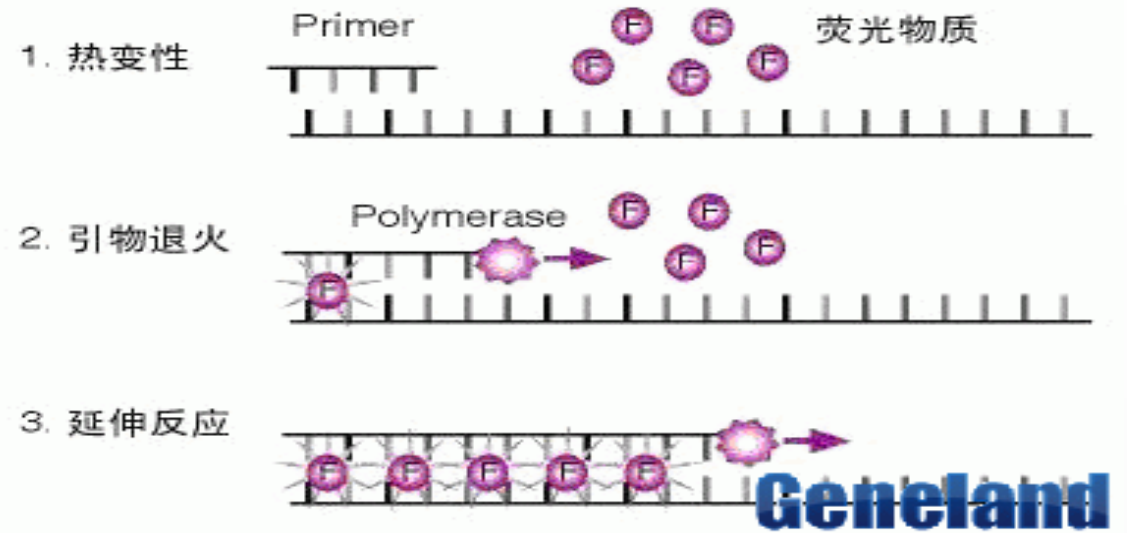


附2：实时荧光定量PCR（real-time quantitative PCR, RQ-PCR）

1. SYBRGreen I 法:

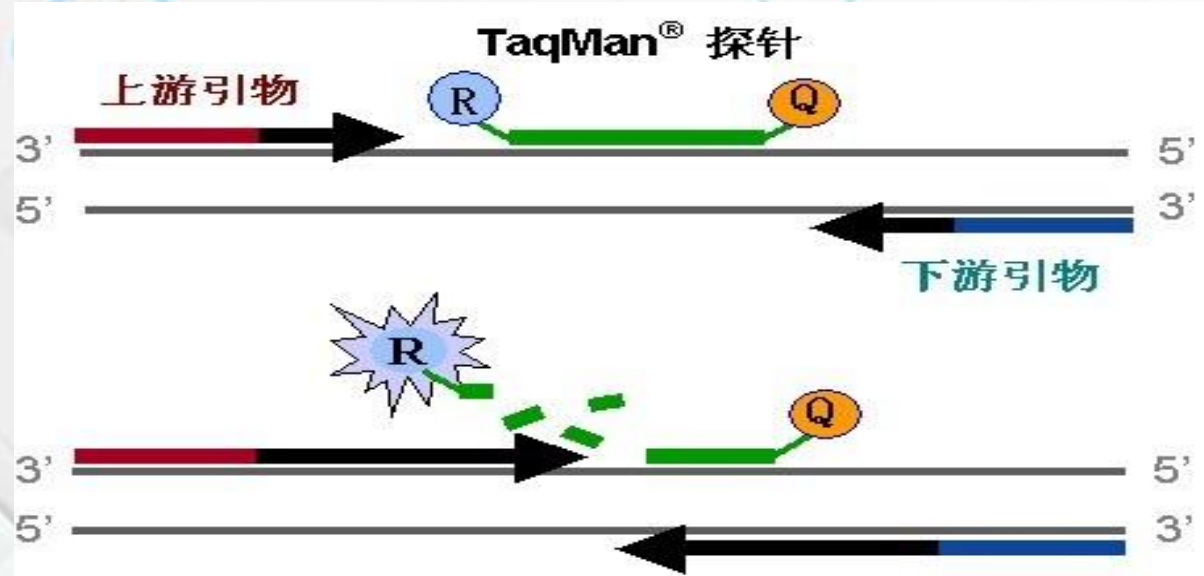
在PCR反应体系中，加入过量SYBR荧光染料，SYBR荧光染料特异性地掺入DNA双链后，发射荧光信号，而不掺入链中的SYBR染料分子不会发射任何荧光信号，从而保证荧光信号的增加与PCR产物的增加完全同步。

PCR产物熔解曲线图（单一峰图表明PCR扩增产物的单一性）



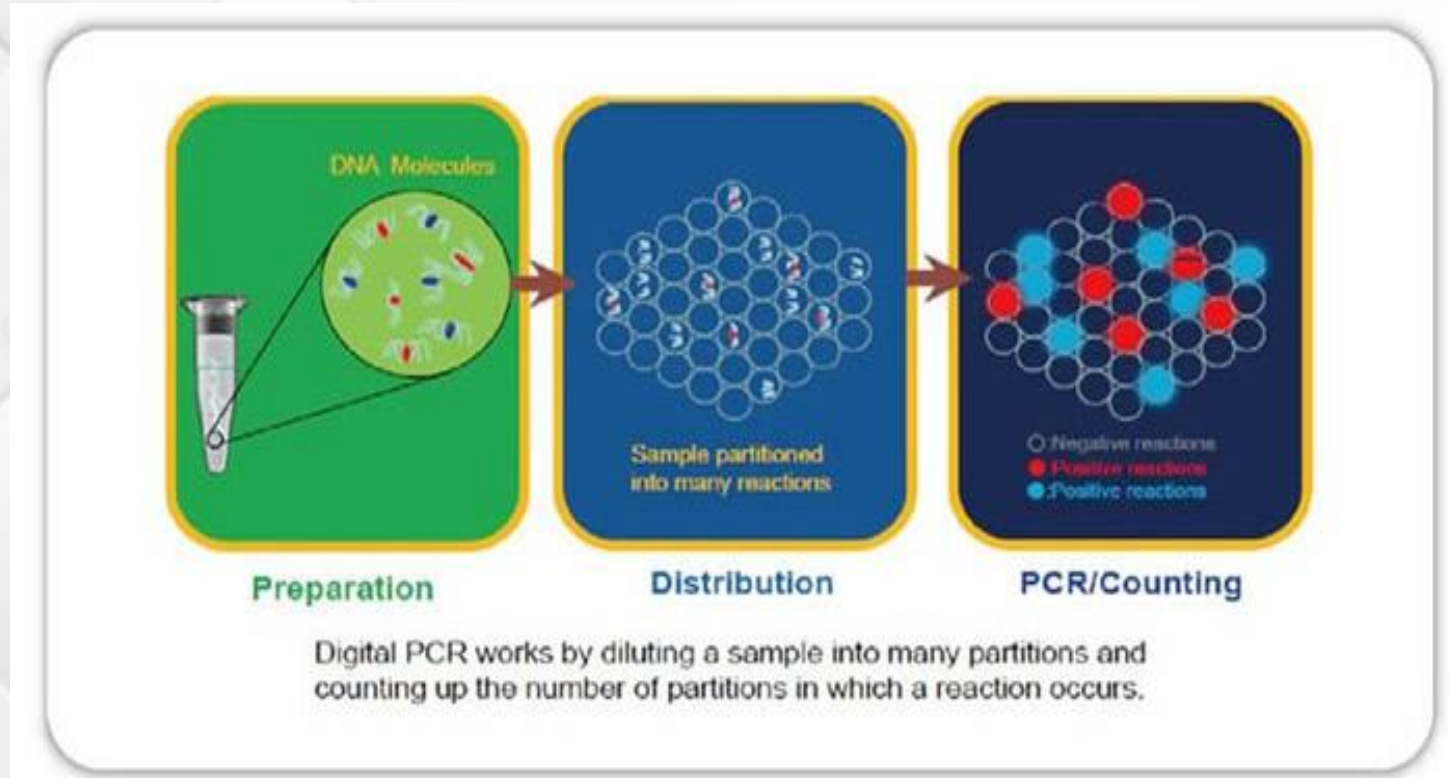
2. TaqMan探针法:

探针完整时，报告基团发射的荧光信号被淬灭基团吸收；PCR扩增时，Taq酶的5'-3'外切酶活性将探针酶切降解，使报告荧光基团和淬灭荧光基团分离，从而荧光监测系统可接收到荧光信号，即每扩增一条DNA链，就有一个荧光分子形成，实现了荧光信号的累积与PCR产物的形成完全同步。



附3：微滴式数字PCR（Droplet Digital PCR, ddPCR）

ddPCR系统在传统的PCR扩增前对样品进行**微滴化处理**，即将含有核酸分子的反应体系分成数万个纳升级的微滴，其中每个微滴或不合待检核酸靶分子，或者含有一个至数个待检核酸靶分子。经PCR扩增后，对**每个微滴的荧光信号**进行逐一分析，有荧光信号微滴判读为1，没有荧光信号微滴判读为0，根据泊松分布原理及阳性微滴的个数与比例即可得出靶分子的起始拷贝数或浓度。





THANKS !