甲基化

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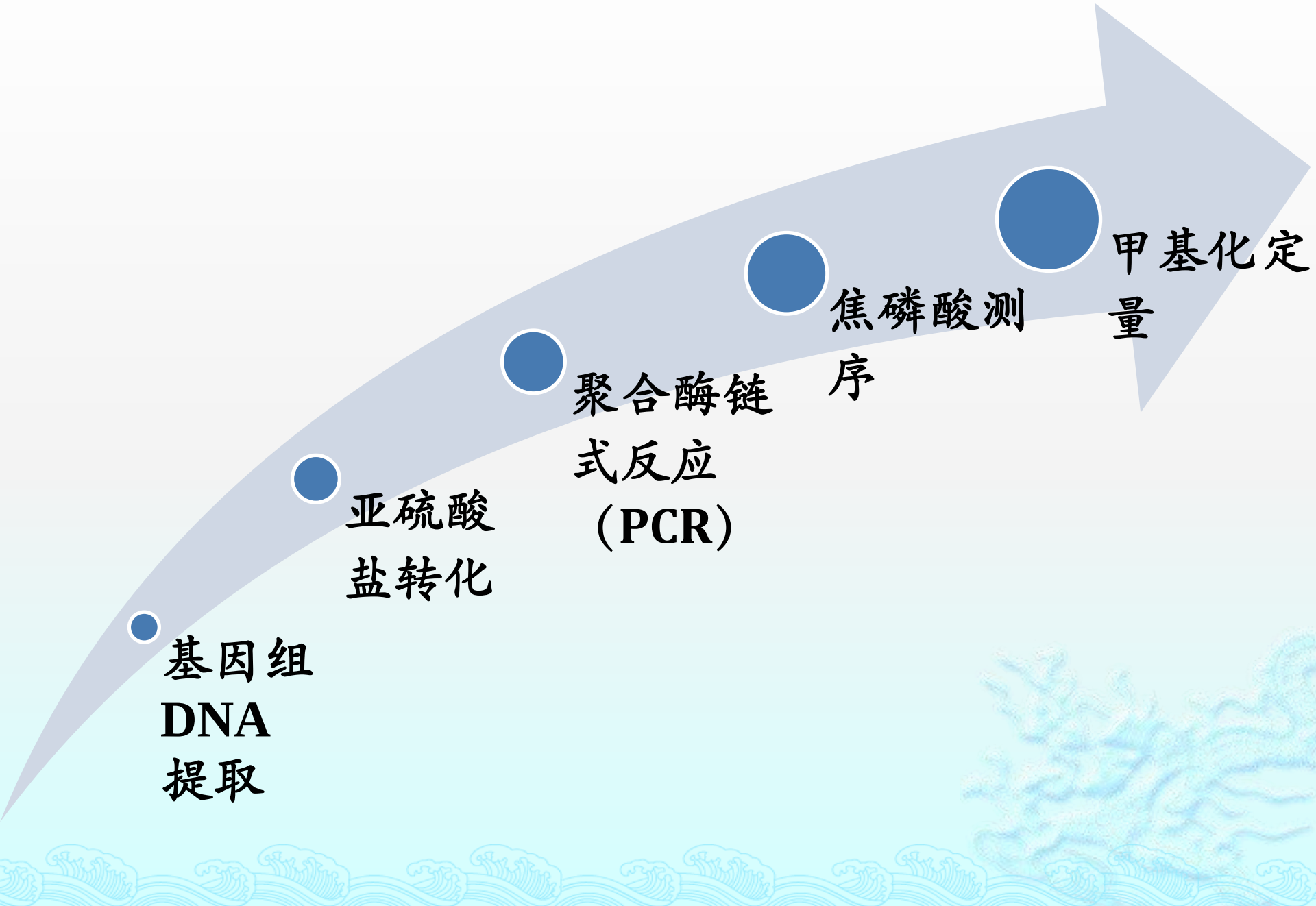

基因组DNA提取
亚硫酸盐转化
聚合酶链式反应（PCR）
焦磷酸测序
甲基化定量

基因组DNA提取

- 试剂盒：基因组DNA小量抽提试剂盒(离心柱式)—碧云天
- 可以抽提动物组织、鼠尾、培养细胞、细菌、酵母、动物血液、昆虫以及固定组织的包括基因组DNA在内的总DNA。

产品编号	产品名称	包装
D0063-1	样品裂解液A	10ml
D0063-2	样品裂解液B	11ml
D0063-3	洗涤液I	21ml (第一次使用前加入7ml无水乙醇)
D0063-4	洗涤液II	16ml (第一次使用前加入24ml无水乙醇)
D0063-5	洗脱液	22ml
D0063-6	蛋白酶K	1.1ml
D0063-7	DNA纯化柱及废液收集管	50套
—	说明书	1份

a. 收集最多不超过500万的细胞，离心沉淀后重悬于200微升PBS中。

b. 清除RNA(可选做)。如果希望获得不含RNA的高纯度总DNA，加入4微升100mg/ml RNase A，Vortex混匀。室温(15- 25℃)放置2分钟。

c. 加入20微升蛋白酶K，Vortex混匀。

d. 加入200微升样品裂解液B，Vortex混匀。70℃孵育10分钟。

e. 加入200微升无水乙醇，Vortex混匀。

f. 把步骤e中的混合物加入到DNA纯化柱内。≥6000g (约≥8000rpm)离心1分钟。倒弃废液收集管内液体。

g. 加入500微升洗涤液I，≥6000g (约≥8000rpm)离心1分钟。倒弃废液收集管内液体。

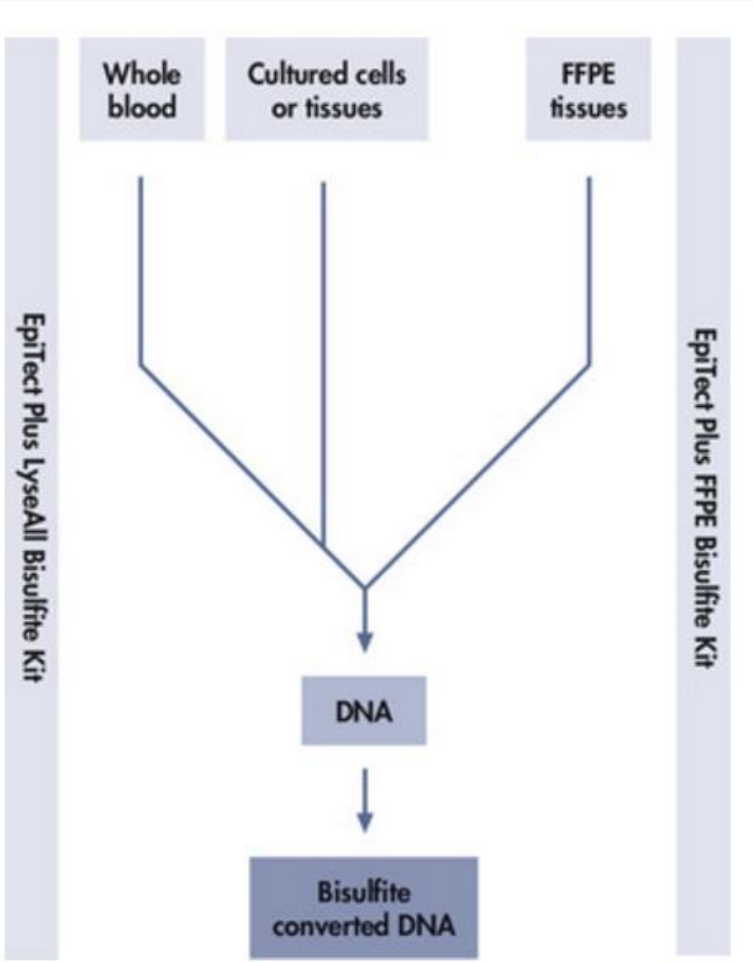
h. 加入600微升洗涤液II，≥18000g (约≥12000rpm)离心1分钟。倒弃废液收集管内液体。

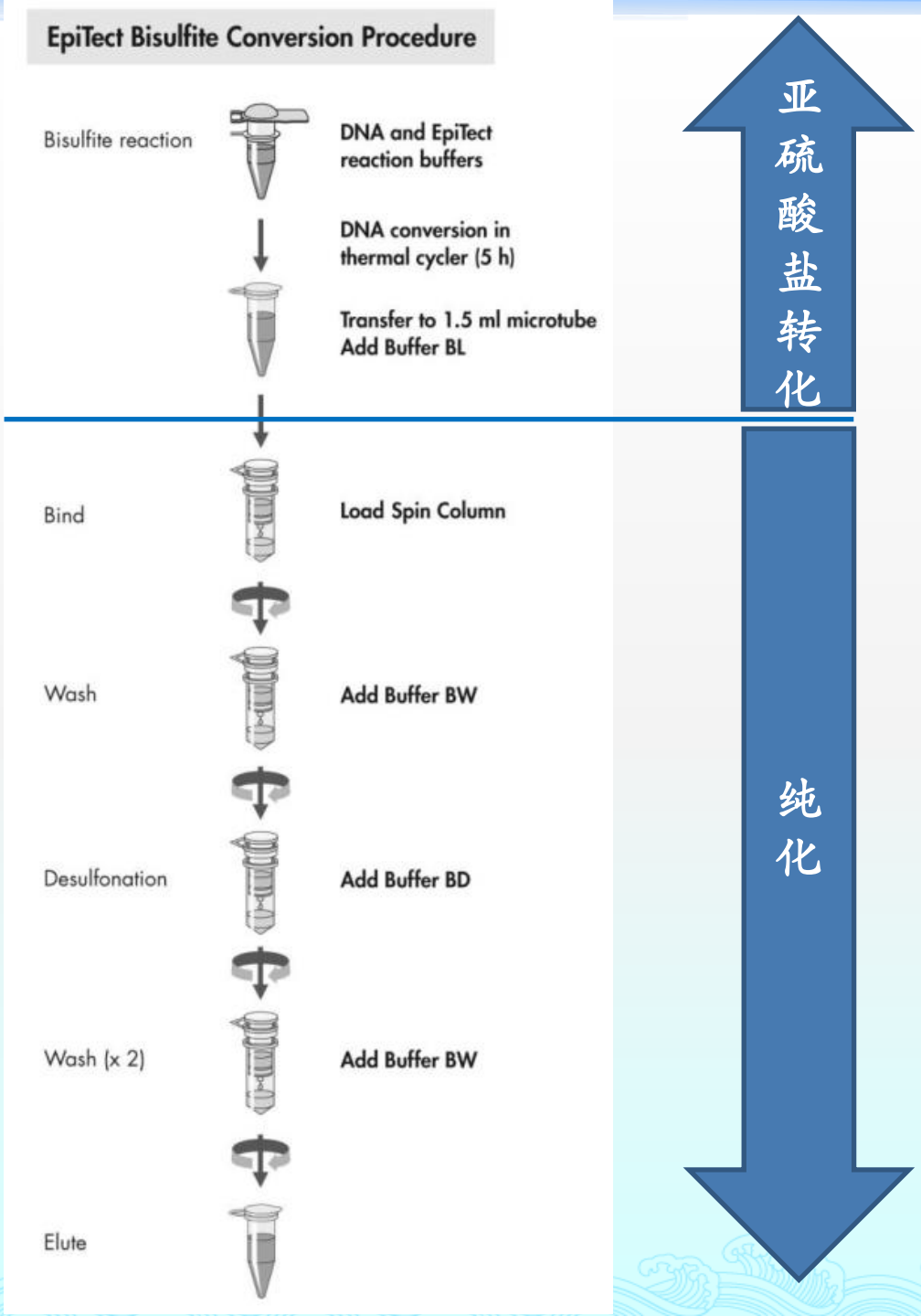
i. 再≥18000g (约≥12000rpm)离心1分钟，以去除残留的乙醇。

j. 将DNA纯化柱置于一洁净的1.5ml离心管上，加入50-200微升洗脱液。室温放置1-3分钟。≥12000rpm离心1分钟。所得液体即为纯化得到的总DNA。

亚硫酸盐转化

- 试剂盒：EpiTect Plus DNA Bisulfite Kit---QIAGEN
- 适用于血液、细胞、组织和石蜡包埋的组织。




EpiTect Bisulfite Conversion Procedure
Bisulfite reaction
DNA and EpiTect reaction buffers
DNA conversion in thermal cycler (5 h)
Transfer to 1.5 ml microtube
Add Buffer BL
Bind
Load Spin Column
Wash
Add Buffer BW
Desulfonation
Add Buffer BD
Wash (x 2)
Add Buffer BW
Elute

亚硫酸盐转化

纯化

DNA亚硫酸盐转化

1. Add 800 µl RNase-free water to each aliquot of Bisulfite Mix needed, and vortex until Bisulfite Mix is completely dissolved. This may take up to 5 min. Dissolving the Bisulfite Mix may require heating the solution to 60°C.

2. Set up the bisulfite reactions in 200 µl PCR tubes according to Table 1. Add each component in the order listed.

Table 1. Bisulfite reaction setup

Component	High concentration samples (1ng – 2 µg) Volume per reaction (µl)	Low concentration samples (1 ng – 500 ng) Volume per reaction (µl)
DNA solution	Variable* (maximum 20 µl)	Variable† (maximum 40 µl)
RNase-free water	Variable*	Variable†
Bisulfite Mix	85	85
DNA Protect Buffer	35	15
Total volume	**140**	**140**

* The combined volume of DNA solution and RNase-free water must total 20 µl.
† The combined volume of DNA solution and RNase-free water must total 40 µl.

DNA亚硫酸盐转化

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. DNA Protect Buffer should turn blue indicating sufficient mixing and correct pH.

4. Program the thermal cycler according to Table 2. Use a cycler with a heated lid.
If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available.

Table 2. Bisulfite conversion thermal cycler conditions

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Incubation	175 min (2 h 55 min)	60°C
Hold	Indefinite*	20°C

DNA纯化

1. For starting material <100 ng DNA, add dissolved carrier RNA to Buffer BL. Prepare only the amount needed for the number of reactions (Table 1).
2. Upon completion of the bisulfite conversion (Protocol 1), briefly centrifuge the PCR tubes. Transfer the reactions to clean 1.5 ml microcentrifuge tubes.
3. Add 310 µl Buffer BL (with 10 µg/ml carrier RNA for <100 ng DNA; step 1) to each sample. Mix by vortexing and then centrifuge briefly.
4. Add 250 µl ethanol (96–100%) to each sample. Mix by pulse vortexing for 15 s and then centrifuge briefly to remove drops from inside the lid.
5. Place MinElute® DNA spin columns and collection tubes in a rack. Transfer the entire contents of each tube (step 4) to a corresponding spin column.
6. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

Table 1. Carrier RNA and Buffer BL volumes

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	350 µl	1.4 ml	2.8 ml	5.6 ml	8.4 ml	16.8 ml
Volume of carrier RNA solution*	3.5 µl	14 µl	28 µl	56 µl	84 µl	168 µl

* Volumes give10 µg/ml carrier RNA in Buffer BL with a 10% surplus for pipetting inaccuracies.

DNA纯化

7. Add 500 µl Buffer BW to each spin column. Centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

8. Add 500 µl Buffer BD to each spin column, close the spin column lids, and incubate for 15 min at room temperature (15–25°C).
Important: Minimize exposure of Buffer BD to air to prevent acidification.

9. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

10. Add 500 µl Buffer BW to each spin column. Centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

11. Repeat step 10.

12. Add 250 µl ethanol (96–100%) to each spin column and centrifuge at maximum speed for 1 min.

DNA纯化

13. Place the spin columns into new 2 ml collection tubes and centrifuge at maximum speed for 1 min to remove any residual liquid.

    Optional: Incubate the spin columns on a heating block at 60°C for 5 min to evaporate the liquid.

14. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Add 15 µl Buffer EB directly onto the center of each spin-column membrane and close the lids gently.
    Note: As little as 10 µl Buffer EB can be used for elution.

15. Incubate the spin columns at room temperature for 1 min.

16. Centrifuge for 1 min at 15,000 x *g* (12,000 rpm) to elute the DNA.

    Note: Store purified DNA at 2–8°C for up to 24 h. For longer storage, we recommend storage at –20°C.


310μl Buffer BL
250μl无水乙醇
转移到spin column
12000rpm离心1min
500μl Buffer BW
12000rpm离心1min
500μl Buffer BD
室温孵育15min
500μl Buffer BW
12000rpm离心1min
250μl无水乙醇
12000rpm离心1min
转移到新的
1.5ml EP管中
15μl Buffer EB
室温孵育1min
12000rpm离心1min

聚合酶链式反应（PCR）

试剂盒：PyroMark PCR Kit (200)---QIAGEN

聚合酶链式反应（PCR）

PyroMark PCR Kit	**(200)**	**(800)**
Catalog no.	**978703**	**978705**
Reaction volume	**25 µl**	**25 µl**
PyroMark PCR Master Mix, 2x *	3 x 0.85 ml	12 x 0.85 ml
CoralLoad® Concentrate, 10x	1 x 1.2 ml	4 x 1.2 ml
Q-Solution®, 5x	1 x 2 ml	4 x 2 ml
$MgCl_2$, 25 mM	1 x 1.2 ml	4 x 1.2 ml
RNase-Free Water	1 x 1.9 ml	4 x 1.9 ml
Handbook	1	1

PyroMark Assay Design Software 2.0

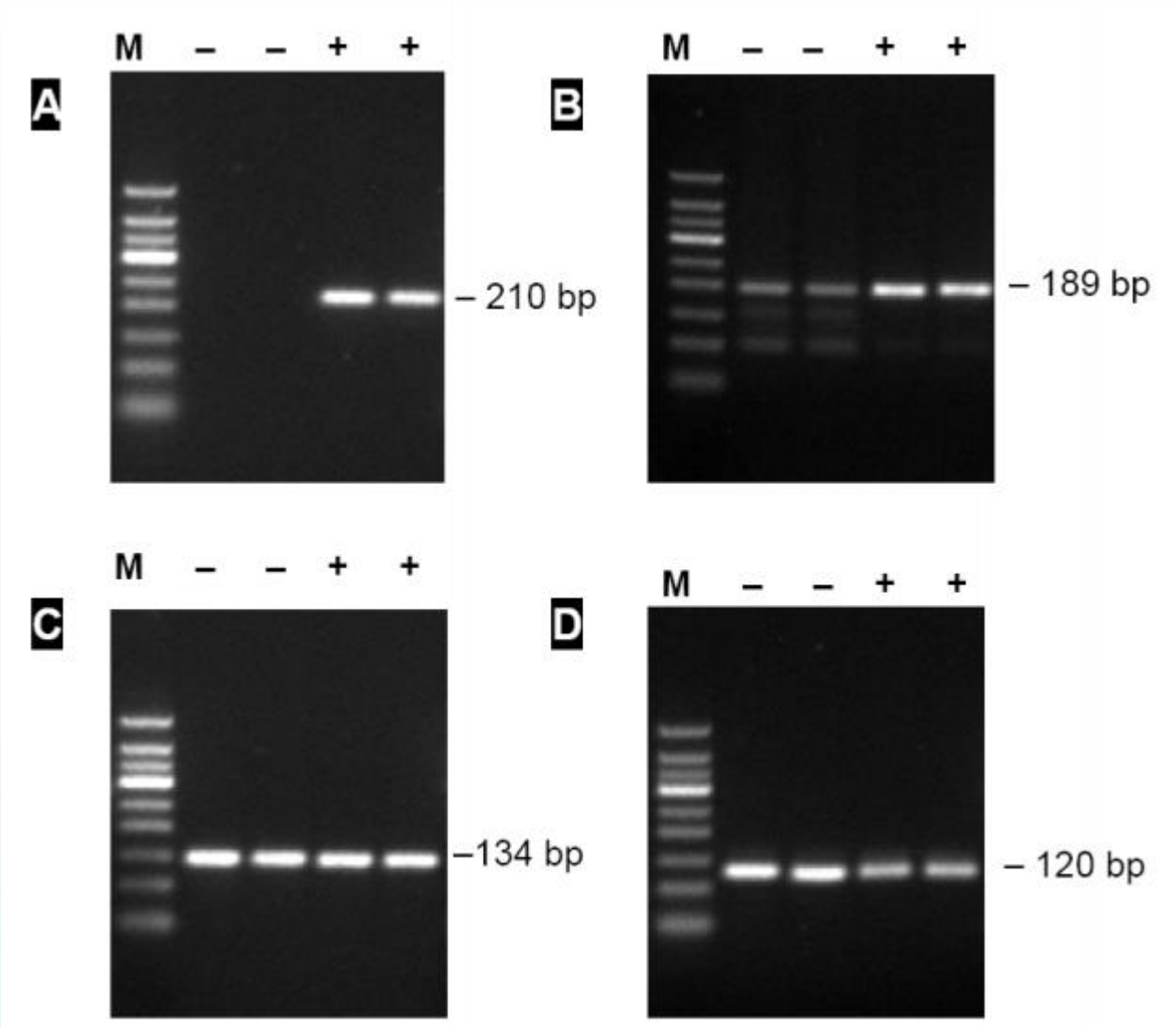
聚合酶链式反应（PCR）

Table 1. Reaction composition using PyroMark PCR Master Mix

Component	Volume/reaction	Final concentration
Reaction mix		
PyroMark PCR Master Mix, 2x	12.5 µl	Contains HotStarTaq DNA Polymerase, 1x PyroMark PCR Buffer,* and dNTPs
CoralLoad Concentrate, 10x	2.5 µl	1x
25 mM $MgCl_2$ (optional)	Variable, see Table 2	See Table 2
Primer A	Variable	0.2 µM†
Primer B	Variable	0.2 µM†
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤ 500 ng/reaction‡ or 10–20 ng bisulfite converted DNA
Total volume	25 µl	

Table 2. Final Mg^{2+} concentrations

Final Mg^{2+} concentration in reaction (mM)	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Required volume of 25 mM $MgCl_2$ per reaction (μl):	0	0.5	1	1.5	2	2.5	3	3.5



-: without Q-solution

+: with Q-solution

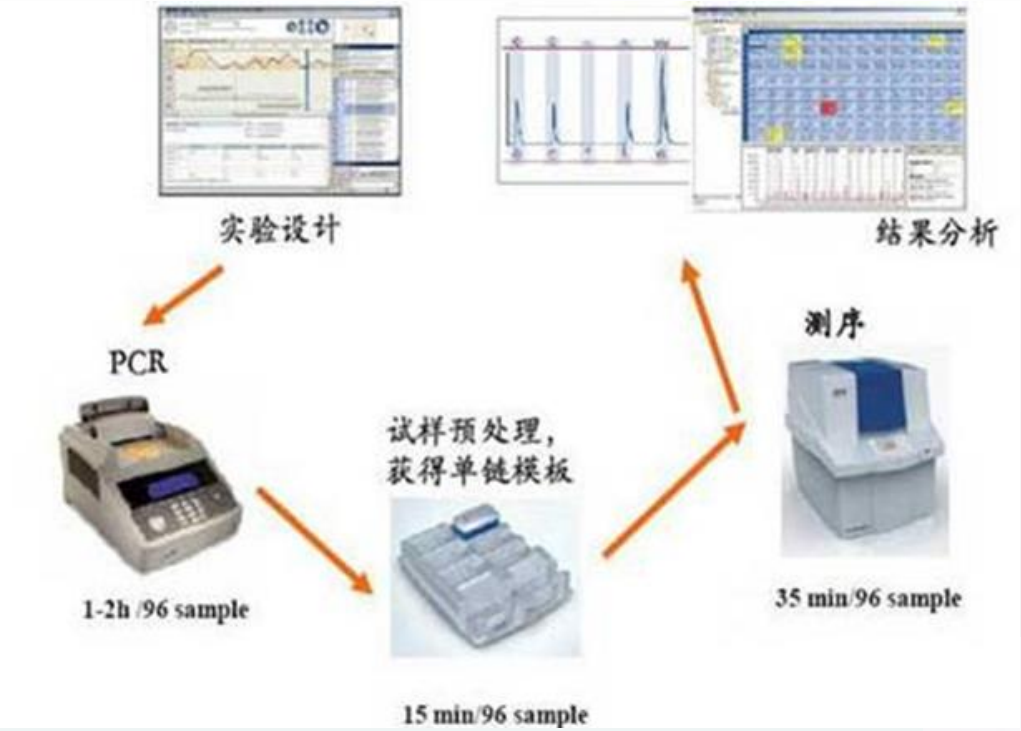
M: marker

聚合酶链式反应（PCR）

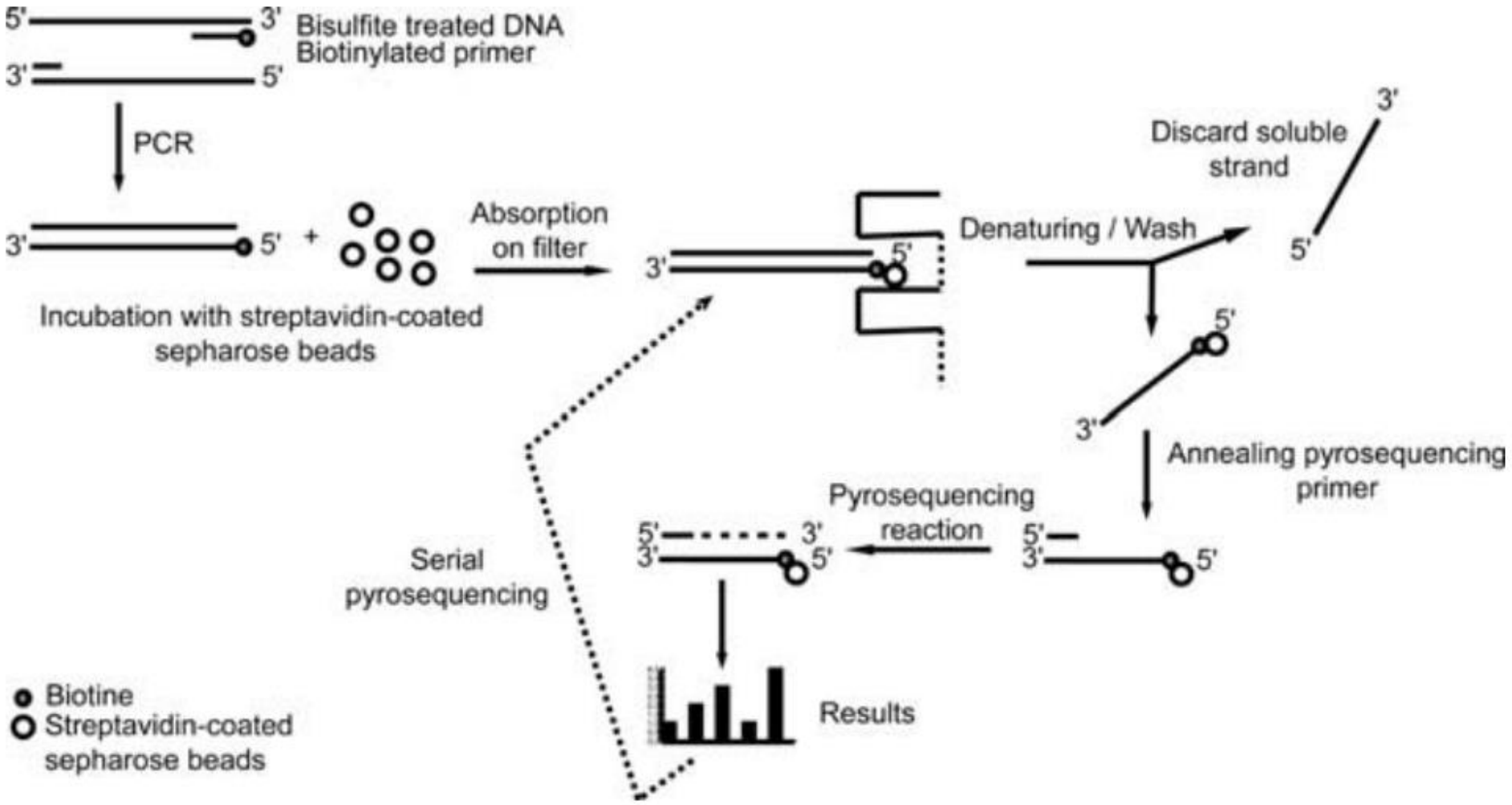
Table 3. Optimized cycling protocol when using PyroMark PCR Master Mix

			Additional comments
Initial PCR activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step
3-step cycling:			
Denaturation	**30 s**	**94°C**	
Annealing*	**30 s**	**60°C**	For genomic DNA
		56°C	For bisulfite converted DNA
Extension	**30 s**	**72°C**	
Number of cycles	**45**		
Final extension	**10 min**	**72°C**	

焦磷酸测序


实验设计
结果分析
PCR
测序
试样预处理，
获得单链模板
1-2h /96 sample
35 min/96 sample
15 min/96 sample

焦磷酸测序


5'
3'
Bisulfite treated DNA
Biotinylated primer
3'
5'
PCR
3'
5'
+
Absorption
on filter
Incubation with streptavidin-coated
sepharose beads
3'
5'
Denaturing / Wash
Discard soluble
strand
3'
5'
5'
3'
Annealing pyrosequencing
primer
Pyrosequencing
reaction
5'
3'
5'
3'
5'
3'
5'
3'
Serial
pyrosequencing
Results
Biotine
Streptavidin-coated
sepharose beads

焦磷酸测序—样品预处理，获得单链模板

1. Transfer 5 µL of the PCR product into a new PCR plate, add 40 µL of binding buffer and 2 µL sepharose beads and complete to 80 µL with water. Cover the plate with a sealing tape and incubate the reaction mixture for 10 min at room temperature under constant mixing (1400 rpm). It is crucial that the beads do not sediment.

2. During this incubation step, prepare the pyrosequencing plate by diluting 4 pmol of the pyrosequencing primer into 12 µL of annealing buffer into the respective wells of the PSQ plate. One or several different pyrosequencing primers can be used on the same plate.

焦磷酸测序—样品预处理，获得单链模板

3. Fill the four troughs of the vacuum preparation tool with 180 mL of 70% ethanol, washing buffer, and water, respectively, and the trough for the denaturing solution with 120 mL 0.2 M NaOH. This different level assures that the NaOH is completely washed off in the following washing step as it might otherwise inhibit the ensuing pyrosequencing reactions.

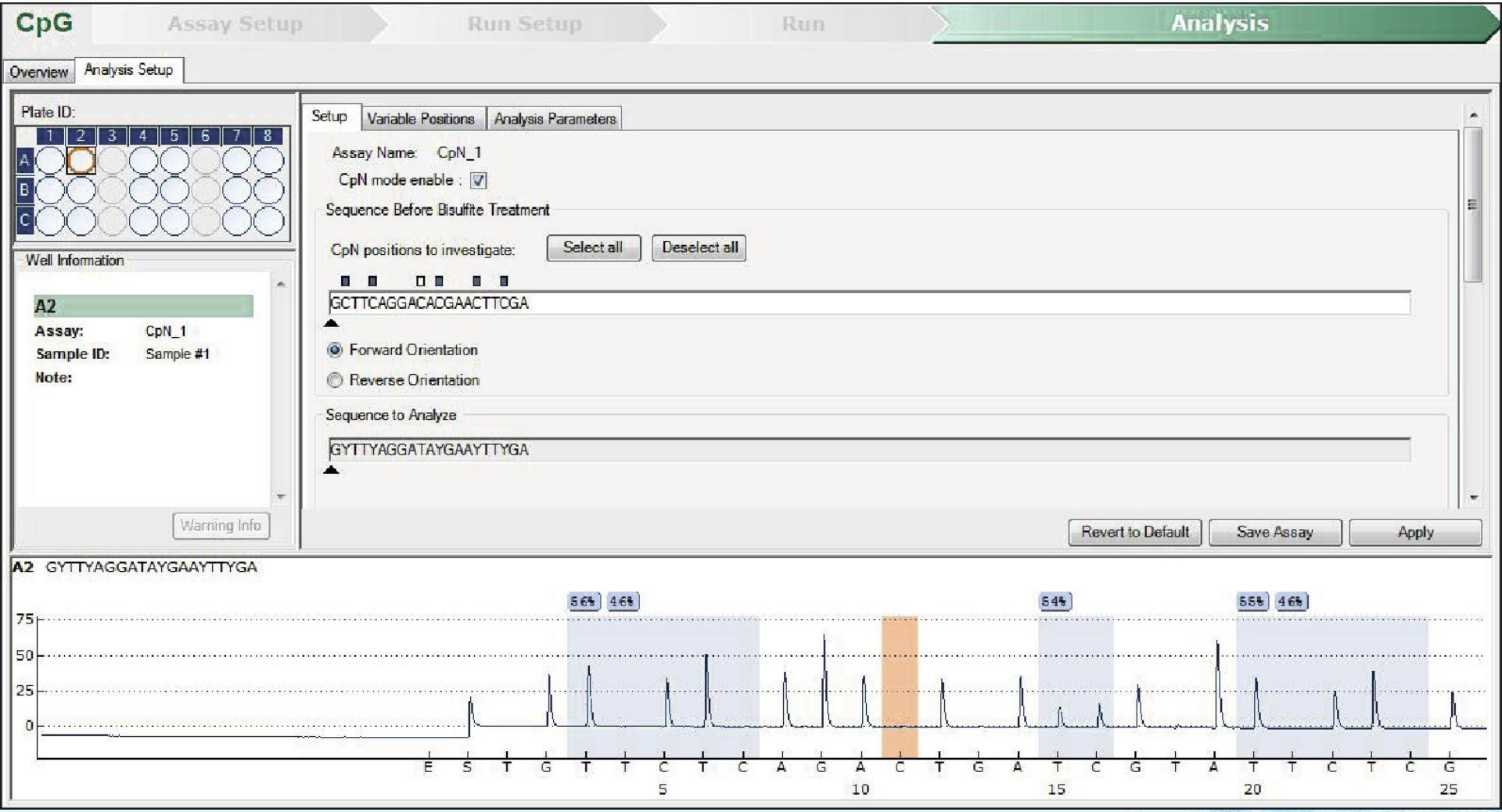
4. Turn on the workstation, create vacuum in the aspiration device (450 mm Hg), and clean the tips of the filters by immersion in water for several seconds. Remove the PCR plate from the mixer and aspirate the binding mix.

5. Immerse the tips of the filters for 5 s each in the successive baths of ethanol 70%, denaturing solution, and washing solution. Turn over the tool and release the vacuum.

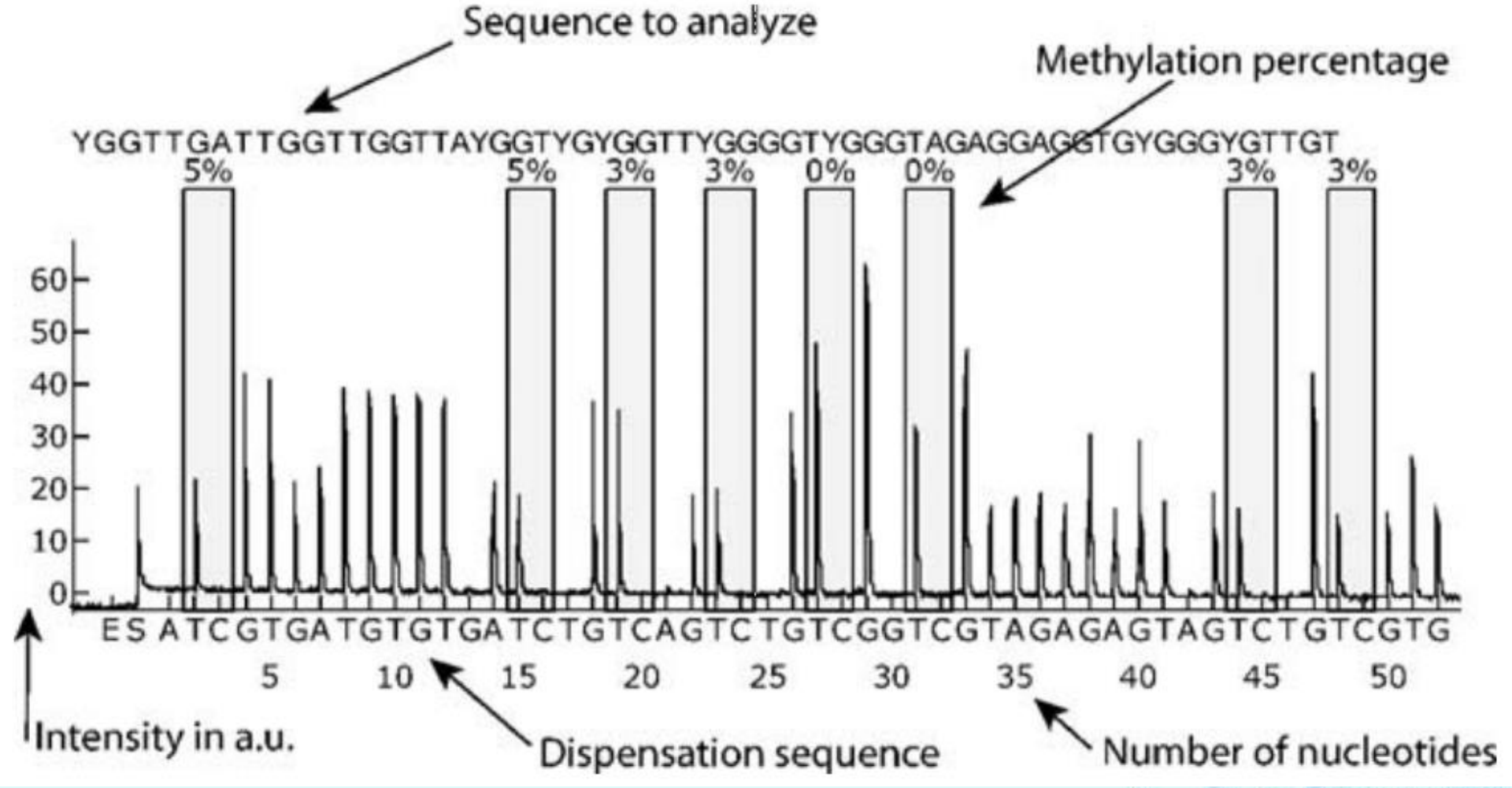
焦磷酸测序—样品预处理，获得单链模板

6. Immerse the tip of the filters in the annealing mix of the sequencing plate and shake gently to release the beads into the wells.
7. Check the plate used for incubation with the binding buffer for the presence of remaining beads. The presence of beads indicates incomplete retrieval of the PCR product which might lead to failure of the subsequent pyrosequencing analysis due to insufficient quantity of template material.
8. Prepared plates can be stored at this point for 2 days at 4◦C prior to pyrosequencing analysis.
9. Incubate the sequencing plate for 2 min at 80◦C on the thermo plate placed on a heating device. Sealing of the plate is not necessary. Allow the plate to cool down to room temperature. For a template-preparation protocol compatible with analysis on the PSQ96MA instrument, please follow the above described procedure and refer to Note 12 for changes.

焦磷酸测序


CpG
Assay Setup
Run Setup
Run
Analysis
Overview
Analysis Setup
Plate ID:
1 2 3 4 5 6 7 8
A
B
C
Well Information
A2
Assay: CpN_1
Sample ID: Sample #1
Note:
Warning Info
Setup
Variable Positions
Analysis Parameters
Assay Name: CpN_1
CpN mode enable :
Sequence Before Bisulfite Treatment
CpN positions to investigate:
Select all
Deselect all
GCTTCAGGACACGAACTTCGA
Forward Orientation
Reverse Orientation
Sequence to Analyze
GYTTYAGGATAYGAAYTTYGA
Revert to Default
Save Assay
Apply
A2 GYTTYAGGATAYGAAYTTYGA
56%
46%
54%
55%
46%
75
50
25
0
E S T G T T C T C A G A C T G A T C G T A T T C T C G
5
10
15
20
25

焦磷酸测序


Sequence to analyze
Methylation percentage
YGGTTGATTGGTTGGTTAYGGTYGYGGTTYGGGGTYGGGTAGAGGAGGTGYGGGYGTTGT
5%
5%
3%
3%
0%
0%
3%
3%
60
50
40
30
20
10
0
ES ATCGTGATGTGTGATCTGTCAGTCTGTCGGTCGTAGAGAGTAGTCTGTCGTG
5
10
15
20
25
30
35
40
45
50
Intensity in a.u.
Dispensation sequence
Number of nucleotides

焦磷酸测序

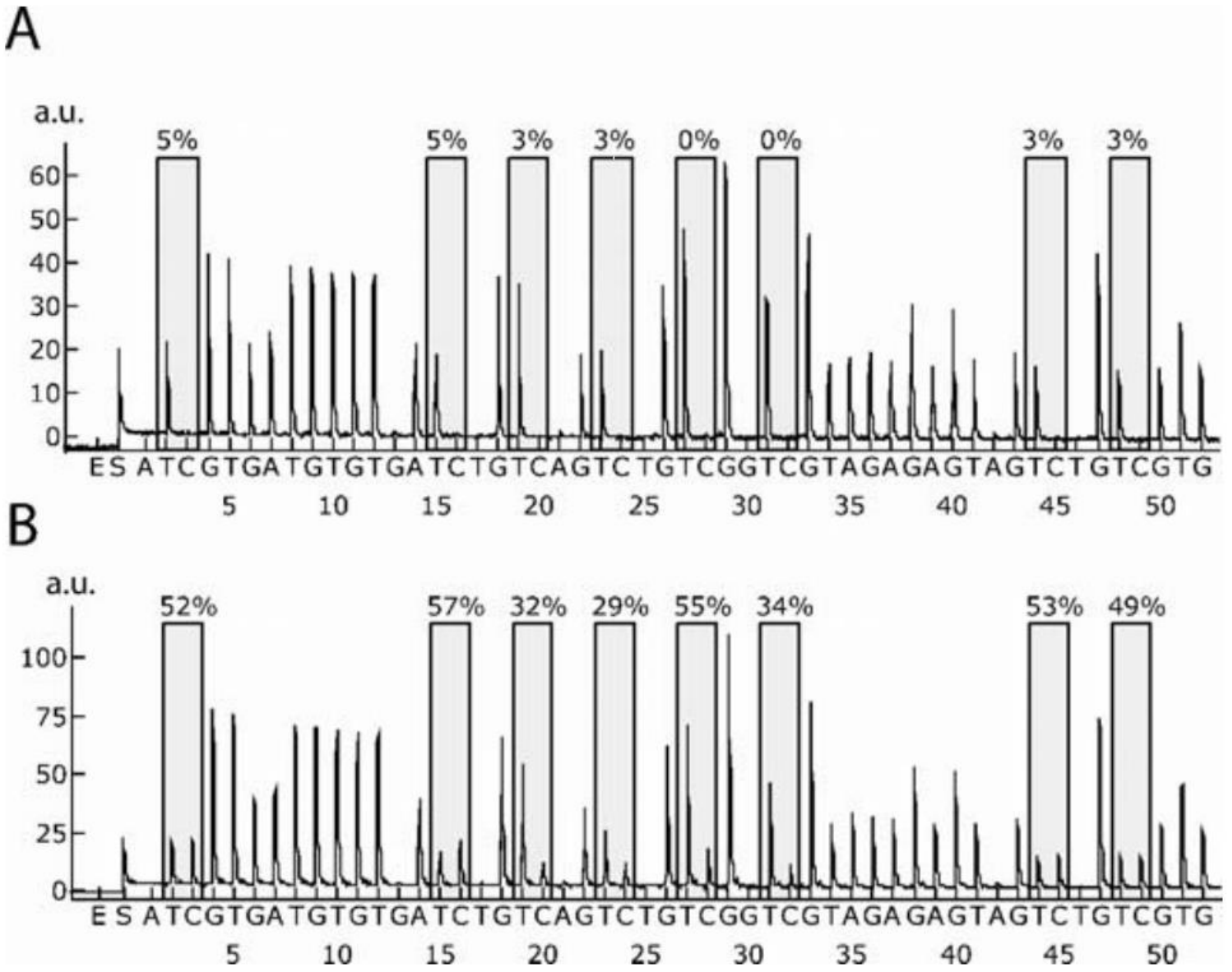


Fig. 15.4. Analysis of DNA methylation patterns in the *CDKN2A* promoter in human hepatocellular carcinoma. Panel A depicts the peritumoral liver tissue corresponding to the tumor shown in panel B. Hardly any methylation is detected in the peritumoral tissue (average 2.7%) while on average 45% methylation is found in the tumor.

Thank you!