Protocols

Rare allele enrichment and detection by allele-specific PCR, competitive probe blocking and melting analysis

Luming Zhou[1], Denice Smith[2], Carl Wittwer[1,2]
[1]*Department of Pathology, University of Utah, Salt Lake City, UT, USA*
[2]*ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, USA*

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Differential amplification of variant and wild-type alleles by PCR is often used for rare allele enrichment. We have combined allele-specific PCR, competitive probe blocking, asymmetric PCR, and melting analysis to enhance rare allele detection in a homogeneous system. Unlabeled, dual hybridization or molecular beacon probes were used for competitive blocking of the wild-type allele at a concentration 10 times that of the allele-specific primer. Probes that both block wild-type amplification and detect rare variants by melting analysis improve the detection sensitivity of allele-specific PCR for rare alleles. In particular, melting analysis using unlabeled probes and amplification by rapid-cycle PCR provides cost-effective and fast enrichment and detection of rare alleles.

Legend

⇨*ATTENTION*

Procedure

Control Sample DNA Extraction

1. Blood, cell lines and frozen normal tissues: Extract genomic DNA using Qiagen DNA purification kits or other standard methods.

Tumor Cell Enrichment and DNA Extraction

2. Frozen tumor tissue: Extract genomic DNA using Qiagen DNA purification kits or other standard methods.

3. Needle rinse specimens: Extract DNA using a Qiagen PureGene kit.

4. Patient samples from Diff-Quik stained fine needle aspirate (FNA) slides – tumor cell enrichment and crude DNA extraction

a. After a pathologist indicates the location of tumor cells by marking the coverslip, use the marks and a diamond pen to draw the areas on the bottom of the slide.

b. Remove the coverslip by soaking in 100% xylene, and incubate sequentially for 2 minutes each in 100%, 95%, and 70% ethanol.

c. Destain by incubating in 1% hydrochloric acid in 70% ethanol for 2 min or until destained.

d. Rinse in running tap water (10-15 minutes), and Scott's tap water substitute (5 minutes).

e. Using a sterile scalpel blade, scrape cells from regions marked with a diamond pencil, and transfer into a microfuge tube containing modified TE buffer with Tween (Table 4; 10-25 µl depending on area scraped).

f. Pipette an equivalent volume of buffer over the same area, and aspirate any residual material into the microfuge tube.

g. Pipette another equivalent fresh volume of buffer over the same area, and transfer into the labeled microfuge tube. Repeat if needed to remove remaining material from area.

⇨***ATTENTION:*** *For FNA scrapes with low numbers of tumor cells aim for a total volume of ~50 µl or less depending on area scraped.*

h. Add 10-20 µl Proteinase K (depending on area scraped and tumor cells present) to microfuge tube and incubate 12-16 hours at 65°C.

i. Centrifuge at 12,000 rpm for 5 minutes, and transfer supernatant to clean screw-capped microfuge tube.

j. Tightly cap the tube, and incubate at 95°C for 10 minutes to inactivate Proteinase K.

5. Patient samples from formalin fixed paraffin embedded (FFPE) slides – tumor cell enrichment and crude DNA extraction.

a. Cut two slides from sequential regions of the tumor cell block, and stain one with hematoxylin and eosin by standard procedures.

Table 1. Preparation Before PCR

Allele-specific primer	5′- GTGATTTTGGTCTAGCTACAGA-3′
Reverse primer	5′-TCAGTGGAAAAATAGCCTCAATTC-3′
Unlabeled probe	5′-TCTAGCTACAGTGAAATCTCGATG-P-3′
Dual hybridization probes	AGCTACAGTGAAATCTCGATGGAG-Fluorescein
	LCRed640-GGTCCCATCAGTTTGAACAGTTGTCTGGA-P
Molecular beacon probe	FAM-CGGTCTAGCTACAGTGAAATCTCGACCG-BHQ1

b. Either stain the second slide with aniline blue or leave unstained.

c. Deparaffinize in xylene, followed by rehydration in ethanol series (100%, 95%, 70%, 50%) and molecular grade water.

d. If staining, incubate in 2.5% aniline blue for 2 minutes.

➪*ATTENTION: Do not coverslip.*

e. Wash in tap water for 2 minutes.

f. Air dry.

g. After a pathologist has marked the coverslip of the H&E slide to indicate the tumor-rich areas, use the H&E slide to identify the same regions on the unstained or aniline blue slide, drawing the areas on the bottom of the slide with a diamond pencil.

h. If using an aniline blue-stained slide, destain by incubating in sterile distilled water.

i. Using a scalpel blade, scrape away the tissue not part of the area of interest.

j. Holding the slide over a biohazard bag, rinse away the scraped tissue with 1 ml of molecular grade water.

k. Scrape the area of interest into one side, and transfer to a microfuge tube containing 10-50 µl of modified TE buffer with Tween (Table 4; volume used depends on tissue size).

l. Pipette an equivalent volume of modified TE with Tween over the area and transfer residual material into microfuge tube.

m. Add Proteinase K (10 – 40 µl depending on area scraped and tumor cells present and incubate 12-16 hours at 65°C.

n. Centrifuge at 12,000 rpm for 5 minutes, and transfer supernatant to clean screw-capped microfuge tube.

o. Tightly cap the tube, and incubate at 95°C for 10 minutes to inactivate Proteinase K.

Recipes

Preparation Before PCR

6. Synthesize the primers and one of the probe alternatives for enriching and

Table 2. Melting Conditions

	Capillary-based real-time thermocycler	Plate-based real-time thermocycler
Denature	95°C, 1 min	95°C, 3 min
Cycles	80	50
	95°C, 0 sec	95°C, 10sec
	64°C, 4 sec, 20°C/s ramp rate	64°C, 5 sec, 2.2°C/s ramp rate
Melting	55°C to 92°C, 0.2°C/s ramp rate	55°C to 92°C, 0.06°C/s ramp rate

Table 3. 5X PCR Master Mix (1 ml)

Adding order	Component	Volume (µl)	5X Master Mix concentration	Final PCR concentration
1	25 U/µl KlenTaq1*	8	0.2 U/µl	0.04 U/µl
2	4 ug/µl Anti-Taq (mAb)*	11	0.044 µg/µl	0.0088 µg/µl
3	2 M (pH 8.3) Tris	125	250 mM	50 mM
4	dH_2O	121		
5	20 mg/ml BSA	125	2.5 µg/µl	0.5 µg/µl
6	1 M $MgCl_2$	10	10 mM	2 mM
7	10 mM dNTPs	100	1 mM	0.2 mM
8	10X LCGreen Plus	500	5X	1X

*Mix KlenTaq and Anti-Taq first and incubate for 20 minutes at room temperature before adding remaining components.

Table 4. Modified TE Buffer with Tween (100 ml)

Component	Volume (ml)	Final concentration
1 M Tris (pH 8.0)	5	50 mM
0.5 M EDTA	0.2	1 mM EDTA
100% Tween 20	1	1% Tween 20
dH_2O	93.8	

detecting the BRAF mutation p.V600E (see Table 1).

7. Prepare 1 ml of 5× PCR Master Mix

Polymerase chain reaction (PCR)

8. Sensitivity of mutation enrichment

Make 10-fold dilutions of p.V600E DNA in wild-type DNA (Final concentration 500 ng/µl):

Pure mutation DNA
10% mutant DNA
1% mutant DNA
0.1% mutant DNA
0.01% mutant DNA
0.001% mutant DNA

9. PCR Preparation

Prepare a PCR pre-mix that includes everything but DNA. For example to make 20 PCR reactions:

40 µl 5× master mix
20 µl forward primer (0.5 µM)
20 µl reverse primer (5 µM)
20 µl probe (5 µM)
80 µl H2O

10. Add 9 µl PCR mix to 10 capillary tubes or plate wells.

11. Then add 1 µl DNA to each capillary tube or plate well. Specifically:

Add 1 µl positive control dilution (500 ng)
Add 1 µl 500 ng wild-type DNA as negative control
Add 1 µl H2O as no-template control
Add 1 µl unknown DNA samples to each other tubes or wells

12. For PCR and melting conditions on capillary or plate thermocycler, see Table 2.

Data Analysis

13. A probe melting peak at 62°C indicates the presence of the mutation.

The positive control has a melting peak at 62°C
The negative control does not have a melting peak at 62°C
The no-template control does not have a melting peak at 62°C
Unknown samples that have a melting peak at 62°C are p.V600E positive.
Unknown samples that do not have a melting peak at 62°C are p.V600E negative or wild-type

Troubleshooting

Low Mutation Enrichment

Raise the annealing/extension temperature

Low PCR Efficiency

Lower the annealing/extension temperature

Primer Dimers

Decrease annealing/extension time or use a rapid capillary thermocycler
Decrease $MgCl_2$ concentration

Reagents

LCGreen Plus (Idaho Technology)
KlenTaq (Ab Peptides, St. Louis, MO)
Anti-Taq monoclonal antibody (eENZYME, Montgomery Village, MD)
Tris (Sigma)
dNTP (Roche)
BSA (Sigma)
$MgCl_2$ (Sigma)
RNAse-FREE Water (Sigma)
BRAF mutation p.V600E cell line HTB-72 (American Type Culture Collection)

Proteinase K (100 mg/ml, Qiagen)
PureGene DNA Isolation kit (Qiagen)
Scott's tap water substitute (ThermoFisher Scientific)

Equipment

Capillary-based thermocycler

LightCycler1.5 (Roche)
LightCycler2.0 (Roche)
LS32 (Idaho Technology)

Plate-based thermocycler

LC480 (Roche)
Any real-time thermocycler with melting function

Address correspondence to Carl T. Wittwer or Luming Zhou, 15 N Medical Drive, Department of Pathology, University of Utah Medical School, Salt Lake City, UT 84132 USA. E-mail: carl.wittwer@path.utah.edu or luming.zhou@path.utah.edu