BTK PCR PROTOCOL

STEP 1

After you have isolated patient's RNA and continued with cDNA synthesis, you perform a nested PCR. The primers used to amplify four coding regions (PH-TS-SK-KIN) are the following. For further information click HERE.

- PH fragment:

PH upper: 5' – GAGTCCCTCCTTCCAAGTC – 3'

PH lower: 5' – GCCACAACCTTTTTCAGC – 3'

T7PH: (nested sense):

5' – TAATACGACTCACTATAGGGCAAGTCCTGGCATCTCAA – 3'

SP6PH: (nested antisense):

5' – ATTTAGGTGACACTATAGAATCTTCCGGTGAGAACTCC – 3'

- TS fragment:

TS upper: 5' – CGTCTTCTCCCCAACTG – 3'

TS lower: 5' – CCATACTTCACTACCCAAAT – 3'

T7TS (nested sense):

5' – TAATACGACTCACTATAGGATCTTCTCCCCAACTGA – 3'

SP6TS (nested antisense):

5' – ATTTAGGTGACACTATAGAACCCAGCTCCTTCAAG – 3'

- SK fragment:

SK upper: 5' – CTGCGGAAGGGTGATGAA – 3'

SK lower: 5' – CCCCAAAAGCCCAAATGT – 3'

T7SK (nested sense):

5' – TAATACGACTCACTATAGGGTGGCAAATATACAGTGTC – 3'

SP6SK (nested antisense):

5' – ATTTAGGTGACACTATAGAACAGGTCTCGGTGAA – 3'

- KIN fragment:

KIN upper: 5' – ATTTGGGGTAGTGAAGT – 3'

KIN lower: 5’ – TTGTGGAGAAGAAAGT – 3’

T7KIN (nested sense):

5’ – TAATACGACTCACTATAGGGTGAAGAAGCCAAAGT – 3’

SP6KIN (nested antisense):

5’ – ATTTAGGTGACACTATAGGAAAGTAGAACCAAGAA – 3’

Notes: the underline sequences constitute the T7 and SP6 promoters.

The PCR conditions for **PH-TS-KIN** regions are (MJ Research PTC-200 DNA Engine):

94 °C for 2 min

30 cycles of :

- 94 °C for 30 sec
- 55 °C for 45 sec
- 72 °C for 90 sec

72 °C for 5 min

8 °C indefinitely

The PCR conditions for **SK** region are:

94 °C for 2 min

30 cycles of :

- 94 °C for 30 sec
- 57 °C for 60 sec
- 72 °C for 90 sec

72 °C for 5 min

8 °C indefinitely

The PCR conditions for all regions for **nested** PCR are:

94 °C for 2 min

25 cycles of :

- 94 °C for 30 sec

- 55 °C for 45 sec
- 72 °C for 90 sec

72 °C for 5 min

8 °C indefinitely

For **50 µl** PCR reaction we use:

- 5 µl **10x PCR buffer** (500mM KCl , 200mM Tris- HCl pH 8.4, Invitrogen Platinum Taq DNA polymerase kit, Cat.10966-034)
- 1.5 µl 50mM **$MgCl_2$** (included in Invitrogen Platinum Taq DNA polymerase kit)
- 0.4 µl 100mM **dNTPs** mixture (Invitrogen Cat. 10297-018)
- 50 pmol of each **primer**
- 0,4 µl 5U/µl Platinum Taq **DNA polymerase** (included in Invitrogen Platinum Taq DNA polymerase kit)
- 0.5 µl of **cDNA**
- Up to 50 µl **distilled H_2O**

STEP 2. TRANSCRIPTION

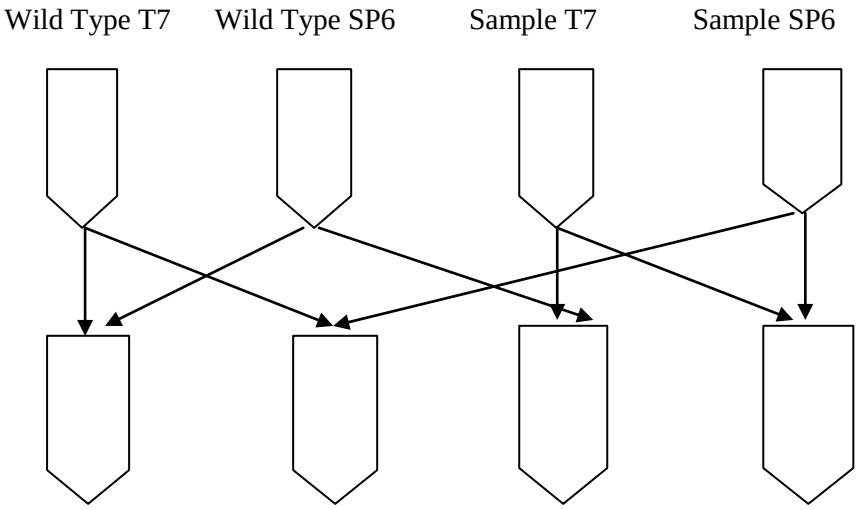
For nested PCR, 1µl of A' PCR product is used as template.

The next step is the transcription of the nested PCR product into single strand specific RNA using T7 and SP6 polymerases. Each sample and one wild type PCR product (per sample), is transcripted separately into 2 different strand specific RNA molecules.

- For reaction volume of **10µl** we use :
  - 4µl distilled H_20
  - 2µl transcription buffer (200mM Tris.Cl, pH 8.0, 40mM $MgCl_2$, 10mM spermidine, 250mM NaCl, stored at -20 °C, Fermentas, included in Fermentas T7 and SP6 polymerase kits)
  - 1µl of 10mM rNTP mixture (Invitrogen Cat. 18109-017)
  - 0.25µl RNase inhibitor
  - 1µl of either T7 (Fermentas #EP 0111) or SP6 polymerase (Fermentas #EP0131)
  - 2µl of PCR product
- Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid
- Incubate at 37 °C for 70min
- Add 10µl of hybridization buffer (25mM EDTA : 25µl of 0.5M EDTA and 475µl DEPC water) (mix gently)
- Briefly centrifuge
- Incubate at 94°C for 3min
- Briefly centrifuge

STEP 3. HYBRIDIZATION

In this step we cross-hybridize the transcription products from the previous step in a fashion shown in this simplified diagram:



- 10µl of each T7 tube is cross-hybridized with 10µl of each SP6 tube (mix gently)
- Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid
- Incubate at 94°C for 3 min. Let hybrids reach room temperature slowly
- Briefly centrifuge

*It has to be noted that the cross-hybridizing procedure should not exceed 8min at room temperature after finishing transcription and before the reaction tubes are returned to 94 °C.

STEP 4. DIGESTION

In this assay, by using RNase 1 and RNase T1, we investigate possible mutation by cleaving mismatches in the RNA hybrids. Each hybridization product is subjected to three different digestion reactions that contain either RNase 1, RNase T1 or a mix of RNase 1 / RNase T1.

- Dilute RNases in digestion buffer (1 mM EDTA pH 8.0, 10mM Tris.HCl pH 7.5, 150mM NaCl, 3mM $CaCl_2$, 50 µg / ml ethidium bromide, stored at -20 °C). Dilutions for each RNase are :
  - RNAse 1 – 1:250 (Ambion Cat. #2294)
  - RNAse T1 – 1:3 (Fermentas #EN0542)

- RNAse mix- 1/1 equal volumes mix from the previously diluted RNAses (1/T1)

- For each RNAse digestion (16µl reaction volume) we use:
  12 µl of RNAse diluted in digestion buffer
  4 µl of hybridization product
- Incubate at 37 °C for 45 min

STEP 5. ANALYSIS OF RNASE CLEAVAGE PRODUCTS BY AGAROSE GEL ELECTROPHORESIS

Add 4 µl of gel loading solution (3M NaCl, 10mM Tris-HCl pH 7.5, 2mM EDTA pH 8.0, 0.25% bromophenol blue, 10µg/ml ethidium bromide, 33% glycerol) that also serves as a stop solution for the digestion process.

Then the product is loaded on 1.5% agarose gel and photographed after approximately 45 mins of electrophoresis at 120V so that each digestion fragment separates adequately from the other ones for the best visible result.