JAK-2 NIRCA PROTOCOL

STEP 1

After you have isolated patient's RNA and continued with cDNA synthesis, perform a nested PCR. The primers used to amplify exon 11-15 JAK-2 coding region are the following. For further information click HERE.

Upper primer

5'- TAA TAC GAC TCA CTA TAG GGA CTG GAC TGT ATG TAC TTC G-3'

Lower primer

5' –ATT TAG GTG ACA CTA TAG GAC CTG TCT TCT TCT CTG ATA AGC-3'

* Nested upper primer has an intergrated T7 promoter and nested lower primer an SP6 promoter.

The PCR conditions for JAK-2 are (MJ Research PTC-200 DNA Engine) :

94 °C for 4mins → initial denaturation

33 cycles of:

- 94 °C for 1 min → denaturation
- 51 °C for 1 min → annealing
- 72 °C for 1 min → extension

72 °C for 5 min → final extension

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)
- 2 μl of **cDNA**
- Up to 50 μl **distilled H_2O**

STEP 2. TRANSCRIPTION

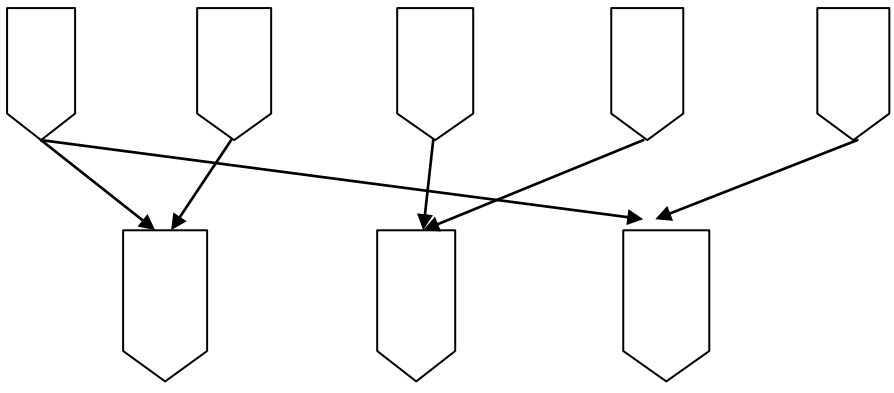
The next step is the transcription of the nested PCR product into single strand specific RNA using T7 and SP6 polymerases. Each sample and one wild type PCR product (per sample), is transcripted separately into 2 different strand specific RNA molecules.

- 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:

Wild Type T7 WTSP6 Sample T7 Sample SP6 Positive SP6



Wild Type T7 Sample T7 Wild Type T7

Wild Type SP6 Sample SP6 Positive SP6

- 10µl of each T7 tube is cross-hybridized with 10µl of each SP6 tube (mix gently)
    1. Wild Type T7 – Wild Type SP6
    2. Sample T7 – Sample SP6
    3. Wild Type – Positive SP6
- 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 8mins 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 1.5 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.

Add 16 µl of each sample and 3 µl of ladder.

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