

5'Race:

(Protocol: Harry Esterbauer)

Primer:

Anchor:

5'-GAC TAC TGT GCT GAG CTG ATG GGI IGG GII GGG IIG GGI G-3'

Td: 92°C

Tm: 80°C

N1:

not necessary in most cases!!!

5'-GAC TAC TGT GCT GAG CTG ATG G-3'

Td: 65°C

Tm: 68°C

GSP1:

typically 15-18 mer, for optimal specificity at annealing temperature of 52°C

GSP2, GSP3:

typically 24-28 mer, for optimal specificity at annealing temperature of 72°C

General Principle:

1. cDNA-first strand:

Superscript, hot start, anneal at upper limit of GSP1 (use short GSP1s for higher specificity, extend at 52°C if possible)

2. RNaseH digestion

2. heat to **denature & spin** to remove primer & NTPs

3. **tail:** TdT (Boehringer Mannheim)

5. heat to **denature & spin** to remove NTPs

5. PCR1:

sense primer: Anchor

antisense: GSP2 (use only GSP2s with high Td/Tm: anneal at 72°C!!!

aliquot1 ad Gel, aliquot2 ad seminested PCR2

7. PCR2:

sense: Anchor

antisense: GSP3

1. cDNA:

RNA (1-3 µg)
 GSP1 (1 pmol)
 dd ad 11.5 µl

70°C, 15'

cycle to annealing temperature: best 52°C (if OK with GSP1)

ad preheated (52°C) mix:

5 x Buffer	4 µl
100 mM DTT	2 µl
10 mM dNTPs	2 µl
RNAasin	0.5 µl
Superscript	1 µl

total volume 20 µl

mix by pipetting

overlay with oil (if no heated lid available)

anneal 5' (RACE works best with 52°C)

extend 52°C, 1 hour

denature 70°C, 15'

freeze

2. RNaseH:

add 1-2 U RNaseH (Boehringer)

37°C, 20'

70°C, 10'

freeze

3. Primer & NTP removal:

use spin column (Pharmacia SR-400 or similar product = size exclusion > primer length)
 if column is preequillibrated with EDTA, then exchange buffer before loading sample!!!

4. Tailing:

concentrate spin-eluate down to 11 µl (speed-vac, use **siliconized** tubes)

add:

RT-reaction	11 µl
2 mM dCTP	1 µl
TdT, CoCl ₂ (Boehringer)	according to manufacturer

dd ad total volume of 20 µl

37°C, 1 hour

70°C, 10'

freeze

5. Buffer & NTP removal:

use spin column (Pharmacia G25/50 or similar - if column is preequillibrated with EDTA, then exchange buffer before loading sample!!!)

6. PCR1

use aliquot of tailing reaction for PCR

if volume (post-spin) is too large, concentrate (speed-vac, use **siliconized** tubes)

primers: Anchor/GSP2 (Td/Tm of GSP2 should be near annealing temperature of 72°C!!! - specificity!!!)

always perform hot start (i.e. add enzyme/buffer at temperature >85°C, always mix well by pipetting w/o removing tube from cycler!!! - avoid any air-bubbles)

perform 2 PCR1s:

1 - 25 cycles

2 - 45 cycles

7. PCR2

= seminested (Anchor/GSP3, Td/Tm of GSP2 should be near annealing temperature of 72°C!!! - specificity!!!)

use 1 µl aliquot of PCR1-1 (25 cycles, after diluting 1/50 - 1/100)

follow same protocol than for PCR1 (= hot start,...)

cycling:

cycle 30 (remove aliquot - 5 µl)

cycle 35 (remove aliquot - 5 µl)

cycle 40 (remove aliquot - 5 µl)

(consecutive removal of aliquots is not an absolute requirement, but may avoid overamplification of unspecific products)

GOOD LUCK, Harry