

## PASE (PolyA-status-examination) analysis (submitted by Bernhard Dichtl)

### A) Ligation

10 $\mu$ l reaction:	2 $\mu$ l	RNA (appr. 500 ng total RNA)
	2 $\mu$ l	PASELinkerOligo (10 $\mu$ M)
	1 $\mu$ l	T4 RNA Ligase Buffer
	1 $\mu$ l	0.1 mM ATP
	1 $\mu$ l	10 $\mu$ g/ml BSA
	1 $\mu$ l	T4 RNA Ligase (10 U) von Fermentas
	2 $\mu$ l	H <sub>2</sub> O

- place at 4 °C o/n (16 hrs)
- store ligation reaction at -20 °C

### B) Reverse transcription

20 $\mu$ l reaction:	1 $\mu$ l	BRevOligo (10 $\mu$ M)
	1 $\mu$ l	dNTPs (10 mM)
	2 $\mu$ l	Ligation reaction (from A above)
	8 $\mu$ l	H <sub>2</sub> O

- heat to 65 °C for 5 min
- quick chill in ice-bath
- quick spin

on ice add the following to each tube:

7 $\mu$ l:	4 $\mu$ l	5x first strand buffer
	2 $\mu$ l	0.1 mM DTT
	0.3 $\mu$ l	RNase Out
	0.7 $\mu$ l	H <sub>2</sub> O

- heat to 42 °C for 2 min
- add 1  $\mu$ l SuperScript II (we usually dilute the enzyme 1:1 with H<sub>2</sub>O right before use)
- incubate for 50 min at 42 °C
- incubate at 70 °C for 15 min
- put on ice
- quick spin

### C) 1<sup>st</sup> PCR

50 µl reaction	1 µl	BRevOligo (10 µM)
	1 µl	transcript specific Upstream Oligo (10 µM)
	1 µl	dNTPs (10 mM)
	10 µl	5x GoTay buffer, brown
	5 µl	25 mM MgCl <sub>2</sub>
	0.5 µl	Go Taq
	2 µl	RT reaction from above
	29.5 µl	H <sub>2</sub> O

PCR conditions: 1x 95°C, 2 min; 30 x 95°C, 15 sec, 45°C, 45 sec, 72°C, 30 sec; final extension at 72°C, 5 min

Analyze 15 µl of PCR product on 1% agarose gel; for abundant RNAs (e.g. GAL transcripts) corresponding PCR products can easily be detected already at this step; however, we usually also observe non-specific bands; to get rid of those, the second, nested PCR has to be performed.

### D) Nested PCR

50 µl reaction	1 µl	ARevOligo (10 µM)
	1 µl	transcript specific nested Oligo (10 µM)
	1 µl	dNTPs (10 mM)
	10 µl	5x GoTaq buffer, brown
	5 µl	25 mM MgCl <sub>2</sub>
	0.5 µl	Go Taq
	2 µl	1:20 dilution (in H <sub>2</sub> O) of PCR reaction from C)
	29.5 µl	H <sub>2</sub> O

PCR: Depending on the abundance of the PCR product following the 1<sup>st</sup> PCR, the optimal number of cycles for the nested PCR has to be determined; we usually do a test PCR and analyze products after 20, 25 and 30 cycles.

PCR conditions: 1x 95°C, 2 min; 20, 25 to 30 x 95°C, 15 sec, 45°C, 45 sec, 72°C, 30 sec; final extension at 72°C, 5 min

Analyze 15 µl of PCR product on 1% agarose gel; following the nested PCR also non abundant transcripts can be detected and there should be no more unspecific bands; in case that non-specific bands appear, adjust PCR conditions or try a different gene specific primer.

## E) pTOPO cloning of PASE PCR products

10 µl:	4.5 µl	nested PCR product
	1 µl	salt solution
	0.5 µl	TOPO vector

- mix gently and incubate for 5 min at RT
- place on ice
  
- transform as follows:
  - add ligation reaction to one aliquot of TOP10F' competent cells
  - incubate on ice for 5 to 30 min
  - heat shock at 42°C for 1 min
  - add 1 ml LB and incubate at 37°C for 30 to 60 min
  - plate on LB/amp/IPTG/Xgal
  - incubate at 37°C o/n
  - pick white colonies
  - make miniprep
  - directly sequence insert

## F) Hot Nested PCR for gel analysis of poly(A) length

50 µl reaction	0.9 µl	ARevOligo (10 µM)
	1 µl	5'-P <sup>32</sup> -ARevOligo spike (100 µM)
	1 µl	Nested Oligo (10 µM; transcript specific)
	1 µl	dNTPs (10 mM)
	10 µl	5x GoTaq buffer, white
	5 µl	25 mM MgCl <sub>2</sub>
	0.5 µl	Go Taq
	2 µl	1:20 dilution (in H <sub>2</sub> O) of PCR reaction from above
	28.5 µl	H <sub>2</sub> O

PCR conditions: 1x 95°C, 2 min; 'optimal' number of cycles 95°C, 15 sec, 45°C, 45 sec, 72°C, 30 sec; final extension at 72°C, 5 min

- add PCR reaction to 6 µl 3M NaOAc pH 5.2 and 2.5 Vol. EtOH
- precipitate at -20°C
- centrifuge at 4°C for 30 min
- take Supp off completely, dry 2 min in SpeedVac
- resuspend pellet in 15 µl formamide loading buffer
- denature for 5 min at 90 °C
- load 7 µl on 6% acrylamide/ 8.3 M urea gel (20x20 cm)
- Marker: 5' end labeled HpaI digested pBR322 fragments (10-20 counts/sec on geiger)
- following run for appropriate time at 700V, dry gel on Whatman 3MM and expose to PI screen o/n

## G) Reagents:

- SuperScript II (200 U/μl; Invitrogen)
- 5x first strand buffer provided by Invitrogen
- RNaseOut (40 U/μl; Invitrogen)
- Go Taq (5 U/μl; Promega)
- 5x Go Taq buffer brown and white and 25 mM MgCl<sub>2</sub> provided with Go Taq by Promega
- T4 RNA ligase (10 U/μl; Fermentas)
- 10x ligase buffer provided by Fermentas
- PASELinkerOligo (5'-phosphate-GATACTACCTCTATGAATTCTTTGCTAGCT  
ACCTGAACTTATCAGACCTAC-3'-amine)
- BRevOligo (GTAGGTCTGATAAGTTCAGGTAGCT)
- ARev Oligo (AGCAAAGAATTCATAGAGGTAGTATC)
- 5'-P<sup>32</sup>-ARevOligo is obtained by T4 PNK labeling of ARevOligo with γ-<sup>32</sup>P-ATP (see e.g. primer extension protocol)
- TOPO TA cloning kit PCR2.1 from Invitrogen (includes all reagents for cloning and competent cells)