RNA extraction, cDNA synthesis and Quantitative PCR for Xenopus and Drosophila samples

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Total RNA extraction of *Xenopus* whole embryos or animal caps using the Absolutely RNA Microprep Kit (Stratagene)

- Take three whole embryos or 8 to 10 animal caps. qPCR works better in early stages (until approx. late gastrula, the expression changes are bigger)
- Collect embryos or caps in a tube, suck off the buffer completely without damaging the embryos, and add 100 µl of Lysis buffer. Vortex immediately and shake for 5 min. Once homogenized, the sample can be kept at -20˚C.
- Extract RNA according to the protocol.
- To increase the RNA yield, use 30 µl of pre-heated (60˚C) Elution buffer, incubate 2 min at RT before spinning, and repeat elution.
- Store RNA samples at -20˚C or -80˚C.

Total RNA extraction of *Drosophila* embryos using the Absolutely RNA Miniprep Kit (Stratagene)

- Cover grape plates used for egg laying with Halocarbon 700 oil. This treatment makes the embryos transparent (after approximately 10 min in oil) and allows one to distinguish live and dead embryos.
- Pick 50 embryos per sample, transfer into 100 µl Lysis buffer, and immediately freeze on dry ice to break the chorion.
- For extraction of total RNA, thaw samples and homogenize them in 0.1 ml glass homogenizers until no intact embryos or pieces are visible.
- Continue with the protocol.

Total RNA extraction of *Drosophila* wing discs using the Absolutely RNA Microprep Kit (Stratagene)

Use 10 wing discs per sample (= 100 µl Lysisbuffer), proceed according to the protocol.

cDNA synthesis using AffinityScript Multi-temp Rev Transcriptase (Stratagene) and random hexamer priming

- mix:
  - 5 – 10 µg total RNA (4 µl of whole *Xenopus* embryo RNA samples, 6 µl of *Xenopus* animal cap RNA samples, and 6 µl of any *Drosophila* RNA samples works well)
  - 4 µl $d[N]_6$ 100 mM
  - x µl H_2O, for reaction volume of 10 µl

  → 65°C 5', slowly cool down to RT, about 20'
- add:
  - 2 µl 10x AffinityScript buffer
  - 2 µl DTT
  - 0.5 µl RNase Inhibitor
  - 3 µl dNTPs (10 mM)
  - 1 µl AffinityScript RT

  → 42°C for about 3 hrs
- measure the concentration. The undiluted cDNA samples should have an OD260 of ≤1. Dilute the sample to ~0.8 µg/µl. Use 1 to 1.3 µl for each qPCR reaction.
- Store cDNA samples at -20˚C.

Quantitative real-time PCR on the Mx3000P (Stratagene) using SYBR Green QPCR Master Mix (Stratagene)

- Calculate the number of samples you want to run. Don't forget the normalizer!!!
- Prepare a tube for the mastermix for each primer
- calculate the mastermixes, pipet everything **except** for the cDNA:

	1 reaction (15 µl)
2x SYBR Green master mix	7.5 µl
ROX (reference dye) 1:500	0.225 µl
Primermix (10µM each)	0.25 µl
H_2O	fill up to 15 µl
Add later:	
cDNA (~0.8 µg/µl)	1 to 1.3 µl

- Prepare the PCR tubes in a rack on ice and label them.
- Aliquot the mastermix into the PCR tubes.
- Add the cDNA.
- Close the tubes, use optical clear caps only. Vortex gently and spin down briefly.
- Select the wells, use SYBR and ROX.
- Start machine only after the warm-up of the lamp is completed (20 min).
- Make sure you click "turn lamp off after run", unless you want to do another run immediately afterwards.

- Use *ODC* (*Ornithine decarboxylase*) as normalizer gene for *Xenopus* samples
- Measurements should be performed in triplicates or quadruplicates.
- Fold change values (x) are calculated using the following formula: $x=2^{-\Delta\Delta Ct}$.
- Standard PCR cycling conditions are: denaturation at 95°C for 30 sec., annealing at 55°C for 60 sec., and extension at 72°C for 30 sec, 40 cycles