Affymetrix Probe Synthesis Guidelines AtGenExpress

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The following protocol was used for all samples contained in the developmental series of AtGenExpress (Tübingen set). The protocol is streamlined for larger sample numbers. It is imperative that you familiarize yourself with the original protocols provided by Affymetrix and by the suppliers of the individual kits before starting probe preparation.

RNA isolation from whole plants and seedlings

Use of “Plant-RNeasy Mini Kit” (QIAGEN) to isolate total RNA is recommended. Make sure you use sterile and nuclease-free glassware or plastic.
Before first use, READ THE MANUAL!!!

Short protocol:

- grind ~50 mg tissue under liquid N2 to a fine powder
- transfer powder to an sterile Eppendorf tube
- add 450μl buffer RLT (containing 10-μl/ml β-Mercaptoethanol) and vortex vigorously
- store sample in liquid N_2; at this point you can process the remaining samples
- thaw samples
- incubate 3 min at 55°C
- apply suspension to a QIAshredder column and centrifuge for 2 min at max. speed
- transfer flow-through to a new Eppendorf tube without disturbing the pellet
- add 0.5 volumes 95% EtOH and mix by pipetting
- apply sample to RNeasy column
- centrifuge for 20 sec at 10,000g
- discard flow-through
- add 700μl buffer RW1 to the column and centrifuge for 20 sec at 10,000g
- transfer column to an new collection tube
- wash column with500μl buffer RPE and centrifuge for 20 sec at 10,000g and discard flow-through
- repeat washing once and discard flow
- spin for an additional 2 min at max. speed to get rid of residual ethanol
- transfer RNeasy column to a 1.5ml collection tube
- elute RNA with 50μl RNAse-free H_2O; let sit on bench for 2 min before centrifugation to aid elution
- repeat elutution of RNA with additional 50μl RNAse-free H_2O to a total of 100μl
- take OD; note concentration and 260/280 ratio
- precipitate by adding 2 μl glycogen (Ambion, 5mg/ml), 8μl sodium acetate (Sigma, 3M, pH 5.2) and 250 μl EtOH (99%) at –20°C O/N

RNA isolation from apices and small tissue samples

- transfer tissue to an Eppendorf tube
  tip: To avoid losing your sample cut a small hole into a sheet of paper and place a tube in the hole. Put the whole thing over a bucket with dry ice and press the tube into the dry ice. If you spill some of the apices, just pick them up from the paper and put them into the tube
- grind tissue extensively using a eppi-pistil (work on dry ice to keep sample frozen)
- add 450µl buffer RLT containing ß-mercaptoethanol
- continue grinding the tissue for 1min
- take the pistil out of the tube; make sure absolutely all tissue remains in the tube
- mix by vortexing
- store sample in liquid N_2; at this point you can process the remaining samples
- thaw samples
- incubate 3 min at 55°C
- apply suspension to a QIAshredder column and centrifuge for 2 min at max. speed
- transfer flow-through to a new Eppendorf tube
- add 0.5 volumes 95% EtOH and mix by pipetting
- apply sample to RNeasy column
- centrifuge for 15 sec at 10,000g
- discard flow-through
- add 700µl buffer RW1 to the column and centrifuge for 15 sec at 10,000g
- transfer column to an new collection tube
- wash column with 500µl buffer RPE, centrifuge for 15 sec at 10,000g and discard flow-through
- repeat washing with RPE and discard flow-through
- spin for an additional 2 min at max. speed to get rid of residual ethanol
- transfer RNeasy column to a 1.5ml collection tube
- elute RNA with 50µl RNAse-free water; let sit on bench for 2 min before centrifugation to aid elution
- repeat elutution of RNA with additional 50µl RNAse-free H_2O to a total of 100µl
- take OD; note concentration and 260/280 ratio
- precipitate by adding 2 µl glycogen (Ambion, 5mg/ml), 8µl sodium acetate (Sigma, 3M, pH 5.2) and 250 µl EtOH (99%) at –20°C O/N

Resuspension of RNA

- spin 30 min 14k rpm at 4°C to collect RNA precipitate
- carefully remove supernatant
- wash with ice-cold EtOH (70%)
- spin 15 min 14k rpm at 4°C
- carefully remove supernatant
- wash with ice-cold EtOH (70%)
- spin 15 min 14k rpm at 4°C
- carefully remove supernatant
- air dry pellet carefully (do not overdry!)
- resuspend RNA in RNAse free water to obtain a concentration of at least 0.5 μg/μl (maximum volume for cDNA synthesis is 10 μl)
- use 1 μl to take OD and ratio.
- RNA can be stored at – 80°C at this point

ds-cDNA synthesis

Use “Superscript double stranded cDNA synthesis kit” (Invitrogen, # 11917-010) for cDNA synthesis. The kit provides all reagents needed for the synthesis of ds-cDNA. RNA spike controls are sold by Affymetrix (Poly A RNA control kit; # 900433)

1st strand cDNA synthesis:

Use 5 μg total RNA in 10 μl.

- incubate at RNA 70°C for 10 min
- chill rapidly on ice
- prepare mastermix from the following reagents for each sample:

1.1μl T7-dT24-oligo (HPLC purified; 100pmol/μl)
4.4μl 5x 1st strand buffer
2.2μl 0.1mM DTT
1.1μl 10mM dNTP mix
2.2μl SSII reverse transcriptase
0,22 μl spike controls (prepare 1:20 stock, then dilute 1:50)

- add 10 μl mastermix to each RNA sample (final volume 20μl), mix gently; if necessary spin down briefly
- incubate at 45°C for 1h

2nd strand cDNA synthesis:

- prepare mastermix from the following reagents for each sample
  - 100μl H_2O (RNase-free)
  - 33μl 5x 2nd strand buffer
  - 3.3μl 10mM dNTP mix
  - 1.1μl *E. coli* DNA Ligase (10U/μl)
  - 4.4μl *E. coli* DNA Polymerase I (10U//μl)
  - 1.1μl *E. coli* RNaseH (2U/μl)
- add 130 μl mastermix to the 1st strand cDNA (20μl) on ice
- mix gently; if necessary spin down briefly
- incubate at 16°C for 2h (don’t let temperature rise above 16°C)

ds-cDNA clean-up

use the “ GeneChip Sample Cleanup Module” from Affymetrix (# 900371), READ the manual!

- add 600 μl cDNA binding buffer the the 150 μl 2nd strand reaction
- vortex to mix (color of mix must be yellow!)
- apply the sample mix to a cDNA Cleanup Spin Column sitting in a 2 ml collection tube
- spin at > 8000g for 1 min
- discard flow through
- add 750 μl cDNA wash buffer (with EtOH !)
- spin at > 8000g for 1 min
- discard flow through and transfer spin column into new 2ml collection tube
- open cap and spin full speed for 2 min to remove residual EtOH
- transfer spin column into new 1.5 ml collection tube
- pipet 14 μl cDNA Elution Buffer directly onto the membrane and incubate for 1 min
- spin full speed for 1 min to elute (average elution volume is 12 μl)
- ds-cDNA can be stored at –80°C at this point

in vitro Transcription

The “BioArrayHighYield Transcript Labeling Kit” (Enzo; also distributed by Affymetrix) is the system of choice to synthesize biotin-labeled cRNA for use with Affymetrix GeneChips.

<u>Short protocol:</u>

- thaw ds-cDNA on ice
- prepare mastermix from the following reagents for each sample

    - 11 μl H_2O nuclease-free
    - 4.4 μl 10x IVT-buffer
    - 4.4 μl 10x Biotin-labeled Ribonucleotide mixture
    - 4.4 μl 10x DTT
    - 4.4 μl 10x RNase Inhibitor
    - 2.2 μl 20x T7 RNA-Polymerase

- add 28 μl mastermix to 12 μl ds-cDNA (final volume 40 μl) mix gently; if necessary spin down briefly
- incubate at 37°C for 5h

cRNA clean-up

use the “ GeneChip Sample Cleanup Module” from Affymetrix (# 900371), READ the manual!

Short protocol:

- add 60 µl water (RNase free) to the IVT reaction and vortex
- add 350 µl IVT cRNA Binding Buffer and vortex
- add 250 µl EtOH (99%) to the lysate and mix well by pipetting – do not centrifuge!
- apply sample mix (700µl) to IVT cRNA Cleanup Spin Column sitting in a 2ml collection tube
- spin at > 8000g for 1 min
- discard flow through
- add 500 µl IVT cRNA Wash Buffer (with EtOH)
- spin at > 8000g for 15 sec
- discard flow through
- add 500 µl 80% EtOH
- spin at > 8000g for 15 sec
- discard flow through and transfer spin column into new 2ml collection tube
- open cap and spin full speed for 2 min to remove residual EtOH
- transfer spin column into new 1.5 ml collection tube
- pipet 11 µl RNase free water directly onto the membrane and incubate for 1 min
- spin full speed for 1 min to elute
- pipet 10 µl RNase free water directly onto the membrane and incubate for 1 min
- spin full speed for 1 min to elute
- use 1 µl (1:100 dilution in water works fine) to take OD for cRNA concentration and 260/280 ratio
- cRNA can be stored at –80°C
- calculate adjusted cRNA yield/concentration:

  adjusted cRNA yield = RNAm - (total RNA * y)

  RNAm = amount of cRNA meassured (µg)
  total RNA = amount of total RNA used for cDNA synthesis (µg); usually 5µg
  y = fraction of cDNA reaction used for in vitro transcription (usually 1)

cRNA fragmentation

- mix:
    - 15 μg cRNA(use adjusted cRNA concentration!) in a total volume of 16 μl
    - 4 μl 5x fragmentation buffer (provided in Cleanup Module)
- incubate at 94°C for 35 min
- chill rapidly on ice
- store 18 μl fragmented cRNA at –80°C, use the remaining 2 μl for gel analysis

Gel analysis

It is a good idea to check the quality of the synthesized cRNA and the fragmentation on an agarose gel before wasting a GeneChip on a bad probe. It is not necessary to run a denaturing RNA gel, but samples should be prepared in a denaturing sample buffer*.

Short protocol:

- add 5μl sample buffer to the following samples:
    - 2 μl cRNA
    - 2 μl fragmented cRNA
- incubate at 65°C for 10 min
- chill on ice
- run the samples side by side on a 2% agarose gel prepared in 1x TAE, use 1kb marker (GibcoBRL) as a standard.

* mix:
0.1μl EtBr (1mg/ml)
1μl 10x MOPS
5μl formamide
2μl formaldehyde (37%)
3μl RNA dye (50% glycerol containing 12.5mg/ml bromphenol blue and xylene cyanole, each)

Preparation of Hybridization cocktail

- prepare mastermix from the following reagents for each sample

96 µl	RNase free water
137.5 µl	2x hybridization buffer (prepared according to Affymetrix)
2.75 µl	Herring sperm DNA (10 mg/ml, Promega)
2.75 µl	acetylated BSA (50mg/ml, Invitrogen)
2.75 µl	control oligo B2 (Affymetrix)
13.7 µl	20x eukaryotic hybridization controls (Affymetrix heated to 65°C for 5 min)

- thaw the remaining 18 µ fragmented cRNA on ice
- add 232 µl mastermix to each fragmented cRNA for a total volume of 250 µl
- hybridization mix can be stored at –80°C

Hybridization

- adjust GeneChip to room temperature
- prehybridize with 300 µl 1x hybridization buffer at 45 °C for at least 10 min
- heat hybridization mix to 99°C for 5 min followed by 5 min at 45°C
- remove prehybridization buffer from GeneChip cartridge
- fill GeneChip cartridge with 200 µl hybridization mix
- hybridize over night at 45°C at 60 rpm

Washing, Staining and Scanning

- remove hybridization mix from GeneChip cartridge and store at –80°C for later use
- fill GeneChip cartridge completely with non-stringent wash buffer (prepared according to Affymetrix)
- wash and stain following Affymetrix protocols or store at 4°C for up to 10 h