Transcriptomics

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Abstract

This chapter presents the current status of microarray and GeneChip tools in the model legume *Medicago truncatula*. After a short introduction into the major tools available, detailed protocols that can be used to obtain expression profiles from cDNA macroarrays, 70mer long oligonucleotide microarrays and Affymetrix *Medicago* GeneChips are listed. These protocols are successfully applied in the author's laboratories to obtain expression profiles from a range of *Medicago truncatula* tissues and growth conditions. Depending on the equipment of expression profiling facilities, modifications might be required to suit individual demands. Therefore, please use these protocols as a starting point and adjust them after consulting experienced personnel at your local facilities.

1 Overview on *Medicago truncatula* DNA array tools

In the frame of different international networks, a range of expression profiling tools have been developed for the model legume *Medicago truncatula* during the past five years (figure 1). As for other species, the field has moved from the community-driven construction of PCR-product based cDNA-macroarrays, cDNA-microarrays, and 70mer oligonucleotide microarrays to commercial Affymetrix GeneChips.

With respect to cDNA macroarrays, a 2.5k macroarray representing ESTs from arbuscular mycorrhizal roots (Liu et al. 2003) and a 6k macroarray from root interactions (Mt6k-RIT, Küster et al. 2004) were developed. Mt6k-RIT as well as DNA arrays based on curated 1k and 6k unigene sets of *Medicago truncatula* (Lohar et al. 2005) were subsequently used to set up microarray printing and hybridization conditions in different laboratories. Based on a collection of ESTs from developing flowers and pods, the Mt6k-RIT microarray was extended to an Mt8k version (Firnhaber et al. 2005).

During the 1st International Conference on Legume Genomics and Genetics in St. Paul, Minnesota in 2002, the *Medicago* community decided to commission the synthesis of a 16k 70mer oligonucleotide collection representing all TCs from the TIGR *M. truncatula* Gene Index version 5 through the Operon company (http://www.operon.com/arrays/omad.php). This collection of probes was recently extended by 384 probes targeted against transcription factors and other regulators. In the frame of the EU project GRAIN LEGUMES (http://www.eugrainlegumes.org/) and a German network on arbuscular mycorrhiza (MolMyk, http://www.genetik.uni-bielefeld.de/MolMyk/), these tools are referred to as Mt16kOLI1 (Hohnjec et al. 2005) and Mt16kOLI1Plus, respectively.

Array definition files including probe sequences and current probe annotations can be obtained for the Mt6kRIT, Mt8k, Mt16kOLI1 and Mt16kOLI1Plus microarrays under accession numbers A-MEXP-80, A-MEXP-84, A-MEXP-85, and A-MEXP-138 at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). Additional array-related information is also available from http://www.cebitec.uni-bielefeld.de/groups/glp/microarrays/ and from Operon (http://www.operon.com/arrays/omad.php)

Since September 2005, a 51k Affymetrix *Medicago* GeneChip has been available, following the earlier development of a Symbiosis GeneChip based on a previous TIGR *M. truncatula* Gene Index (Barnett et al. 2004). The 51k array was developed from 32,167 expressed sequence tags (cDNAs) in the TIGR *Medicago truncatula* gene index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago), 18,733 genes predicted from *M. truncatula* genome

sequencing that were not represented in the gene index, and 1,896 cDNAs from alfalfa (*M. sativa*) that were not found in the *M. truncatula* sequences. The chip also contains probes covering the *Sinorhizobium meliloti* genome.

Due to the rapid evolution of macro- and microarray tools, only the Mt16kOLI1/ Mt16kOLI1Plus 70mer oligonucleotide microarrays and the Affymetrix *Medicago* GeneChips are currently available to the *M. truncatula* community. In addition, targeted macroarrays are a popular and cost-effective alternative for more focussed analyses. We thus decided to focus the experimental section on protocols related to cDNA macroarrays, 70mer oligonucleotide microarrays and the *Medicago* Affymetrix GeneChips.

2 Macroarray Analysis

Overview

The major step in macroarray analysis is the polymerase chain reaction (PCR) amplification of the cloned cDNAs of interest. We routinely use PCR amplification of cloned cDNAs using purified plasmid DNA as the template, but crude lysates also work well in providing good quality PCR products for array analysis (Hegde et al. 2000). Nevertheless, the quality of PCR products needs to be evaluated by agarose gel electrophoresis as only successful PCR reactions with a single dominant band should be arrayed on filter membranes. PCR products may be arrayed on a filter membrane using a variety of ways. We have used a Q-bot (Genetix, Boston, MA) fitted with a 96-pin gravity gridding head to array cDNA inserts on Gene Screen Plus membranes (Tesfaye et al. 2006; Uhde-Stone et al. 2003). It is important to spot each cDNA insert in duplicate on each filter array. Arrayed filters need to be air-dried and stored at room temperature until used.

We routinely extract total RNA from 100 – 200 mg of frozen plant tissue using the RNeasy Plant RNA mini kit (Qiagen, Valencia, CA). The integrity and concentration of extracted total RNA must be checked by UV spectrophotometer absorbance at 260 and 280 nm. The integrity of total RNA can also be checked using the Agilent 2100 Bioanalyzer in a RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA).

For hybridization, radioactively labeled (α-^{32}P dATP) first-strand cDNA targets are synthesized by reverse transcription of total RNA with the Superscript RT II kit (Invitrogen, Carlsbad, CA), oligo(dT)$_{12\text{-}18}$ primer (Sigma, St. Louis, MO) and dNTP mix as described previously (Fedorova et al. 2002; Tesfaye et al. 2006; Uhde-Stone et al. 2004).

cDNA Clone Growth for Macroarrays

Starter bacterial culture

- Streak out glycerol stocks of *Escherichia coli* containing the *Medicago* cDNA of interest in a plasmid vector on LB agar plates supplemented with 50 µg/mL ampicillin.
- Fill each well of a sterile Sarstedt 96-well microtiter plate with 150 µl LB supplemented with 50 µg/mL ampicillin.
- Inoculate a single colony into a single well of the microtiter plate.
- Seal the microtiter plate with AirPore tape sheet (Qiagen) and place microtiter cover on top.

- Incubate the microtiter plate for 16-18 hours in a 37°C incubator (stationary).
- Use 5 µl of the starter bacterial culture for bacterial culture growth in a 96-well block (see below).

* It is recommended to archive an aliquot (50 µl) of the starter bacterial culture in a microtiter plate containing glycerol at –80°C for future use.

Bacterial culture growth

- Fill each well of a 96-well block (2 ml deep well; Qiagen) with 1.2 ml of 2X YT (below) supplemented with 50 µg/mL ampicillin.
- Inoculate 5 µl of each pre-culture into a single well of the 96-well block.
- Seal the 96-well block with AirPore tape sheet (Qiagen).
- Incubate the bacterial culture for 20-24 h at 37°C in a shaking incubator at 250-300 rpm.
- Take the 96-well block out of the shaker and place on ice for 10-15 min.
- Harvest bacterial cells in the 96-well block by centrifugation for 5 min at 3600 rpm using a rotor for a 96-well block.
- Decant the culture medium by inverting the 96-well block over a bio-hazard waste container that contains 10% bleach. (When decanting, invert the 96-well block quickly to minimize any cross contamination between wells).
- Blot any residual medium by placing the inverted 96-well block onto a paper towel. (Use a fresh paper towel for each 96-well block).

Plasmid DNA miniprep step

- Resuspend bacterial cell pellets in 150 µl of solution I (below) containing 100 µg/ml RNase A. Seal the 96-well block with 4" aluminum tape and vortex to resuspend cells.
- Add 300 µl of Solution II (below) to each well. Seal the 96-well block with a new piece of 4" aluminum tape and gently invert the block 4-6 times. Incubate at room temperature for 5 min (begin timing when solution is added to first row).
- Remove tape from the 96-well block and add 225 µl of Solution III (below). Seal the block with a new piece of 4" aluminum tape and invert the block 4-6 times.
- Incubate the 96-well block on ice for 10 min.
- Centrifuge the 96-well block at 3600 rpm for 20 min at 4°C.
- Securely tape a 96-well Polyfiltronics filter (Whatman) on top of a new autoclaved 96-well block so the wells between the filter and the 96-well block line-up perfectly.
- Transfer 600 µl of the lysate to a single well of the 96-well Polyfiltronics flter.
- Centrifuge at 3600 rpm for 25 min at 4°C.
- Remove the Polyfiltronics filter and add 1000 µl of ethanol to each well of the 96-well block sample.
- Seal the 96-well block with a new piece of 4" aluminum tape and mix by inverting several times.
- Centrifuge the 96-well block for 20 min at 3600 rpm.
- Remove the supernatant carefully and add 700 µl of 70% ethanol to each well.
- Centrifuge the 96-well block for 5 min at 3600 rpm.

- Decant ethanol carefully and vacuum dry the 96-well block for 15 min.
- Resuspend DNA by adding 100 µl of sterile distilled water into a single well of the 96-well block.

* Resulting purified plasmid DNA samples are used for DNA sequencing to confirm identity of the cDNA clone of interest.
* For PCR amplification of the cDNA insert, prepare a 1:10 dilution of purified plasmid DNA in sterile distilled water.

Solution I
50 mM glucose
25 mM Tris, pH 8.0
10 mM EDTA, pH 8.0

Solution III (3 M potassium / 5 M acetate), pH 5.5
For 100 ml of Solution III
29.4 g potassium acetate
Dissolve in 50 ml of distilled water
Add glacial acetic acid (~ 29 ml) to adjust pH to 5.5.
Bring volume to 100 ml.

Solution II (Lysis buffer)
Make fresh just before use!!
0.2 N NaOH
1% SDS

2X YT medium (per liter)
Bacto-typtone 16 g
Bacto-yeast extract 10 g
NaCl 5 g
Adjust pH to 7.0 with 5 N NaOH.
Autoclave.

PCR amplification of cDNA clones for macroarray analysis

cDNA inserts to be spotted on macroarray filters are amplified by PCR using the following protocol.

PCR reaction mix for a single PCR reaction in 100 µl

10 X PCR Buffer	10.0 µl
50 mM $MgCl_2$	5.0 µl
T3 Primer (1035 ng/µl)	1.0 µl
T7 Primer (1050 ng/µl)	1.0 µl
dNTP mix (2.5 mM of each dNTP)	8.0 µl
Platinum Taq DNA polymerase (5 U/µl)	0.4 µl
Diluted cDNA plasmid DNA (1:10)	2.0 µl
H_2O	72.6 µl
TOTAL	100.0 µl

* Add the Platinum Taq DNA Polymerase last, taking it directly from the freezer.
* Prepare a master mix when preparing more than 2 PCR reactions.

Place PCR tubes in a thermocycler and run the following cycle conditions:

1 cycle of 94°C for 2 min

30 cycles of:
94°C for 30 sec
52°C for 30 sec
72°C for 2 min

1 cycle of 72°C for 5 min

Use 3 µl of the PCR reaction for electrophoresis in a 1% agarose gel.

Synthesis of radioactively labeled cDNA by reverse transcription

We routinely extract total RNA from 100 – 200 mg of frozen plant tissue using the RNeasy Plant RNA mini kit (Qiagen). The integrity and concentration of extracted total RNA must be checked before this step using standard procedures (above). Radioactively labeled first-strand cDNA targets are synthesized by *in vitro* reverse transcription of 30 µg total RNA (below).

Protocol for the preparation of labeled cDNA targets by reverse transcription:

1. In a 1.5 mL microcentrfuge tube:
   a. Add 30 µg total RNA for each sample.
   b. Add 1 µl of 0.5 µg/µl Oligo dT $_{(12\text{-}18)}$ primer.
   c. Add RNase-free water to a final volume of 7 µl.
   d. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.
   e. Incubate the reaction for 10 min at 70^{0}C.
   f. Cool the sample on ice for at least 2 minutes.
   g. Centrifuge the tube briefly (~5 seconds) to collect the sample at the bottom of the tube.

2. In a separate tube, assemble First-Strand Master Mix
   a. Prepare sufficient First-Strand Master Mix for all the RNA samples. When there are more than 2 samples, it is helpful to aliquot additional material (at least 10% more) to compensate for potential pipetting inaccuracy or solution lost during the process.

The following First-Strand Master Mix is for a single reaction.

Component	Volume
5X First-Strand buffer	4 µl
dNTP mix (see below)	1 µl
0.1 M DTT	2 µl
Superscript II RNase H$^-$ Reverse Transcriptase (200 units/µl)	1.0 µl

b. Mix well by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the master mix at the bottom of the tube.

dNTP Mix
1 µl 0.25 mM dATP
1 µl 10 mM dTTP
1 µl 10 mM dGTP
1 µl 10 mM dCTP

3. Transfer 8 µl of the First-Strand Master Mix to each RNA/Oligo(dT) Primer mix for a final reaction volume of 15 µl.
4. Add 5 µl of α-^{32}P dATP (3,000 mCi mL^{-1}) to each tube to a final reaction volume of 20 µl.
5. Mix by pipetting and spin briefly (~5 seconds) to collect the contents at the bottom of the tube.
6. Incubate the reaction for 1 hour at 42^{0}C.
7. Add 1 µl of 5 mM dATP to each tube and incubate for additional 30 minutes at 42^{0}C.
8. Denature labeling reaction by adding to each reaction tube:
   2 µl 0.5 M EDTA
   3 µl 5 N NaOH
9. Incubate reaction tubes for 30 minutes at 65^{0}C.
10. Neutralize each reaction tube by adding to each reaction tube:
   8 µl 1 M Tris (pH 7.4)
   7 µl 2 N HCl
11. Remove the unincorporated-labeled nucleotides. We successfully used Micro Bio-Spin® Chromatography Columns (BIO-RAD, Hercules, CA) for this task.
12. Remove 1.0 µl of labeled cDNA for scintillation counting.

Hybridization of macrorarray membranes

1. Preparing spotted macroarray membranes for hybridization

We have used a Q-bot (Genetix) fitted with a 96-pin gravity gridding head to array cDNA inserts on Gene Screen Plus membranes (Gentix). Arrayed membranes need to be air-dried and stored at room temperature until used.

a. Place arrayed membrane, cDNA side up, on a pad of three absorbent filter papers (Whatman 3MM) soaked with denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 10 min.
b. Transfer membrane, cDNA side up, on a pad of three absorbent filter papers (3MM) soaked with neutralizing solution (1.5 M NaCl and 1 M Tris pH 8.0) for 5 min.
c. Wash membrane briefly in 2 x SSC with gentle shaking (~1 minute).
d. Transfer membrane, cDNA side up, on to a clean, dry absorbent filter paper to air-dry.
e. Fix macroarray membrane using a UV cross linker at 120 joules cm^{-2}.

2. Hybridization protocol

a. Pre-warm the hybridization oven to 42^{o}C. Warm pre-hybridization solution (below) to 42^{o}C for about 10 min prior to use.
b. Pre-hybridize macroarray membrane with pre-hybridization solution for at least 30 min at 42^{o}C. Use approximately 1 ml for every 10 cm^2 of membrane. Use roller bottles at slow rpm.

c. Decant and discard the pre-hybridization solution from the array. To the array roller bottle, add fresh hybridization solution (below; using 1 ml for every 10 cm^2 membrane) and labeled cDNA adjusted to 10^6 cpm mL^{-1}.
d. Hybridize for 12-18 hrs at 42°C.
e. Decant hybridization solution into radioactive liquid waste.
f.. Add wash Solution I (below; pre-warmed to 42°C) to the roller bottle (use at least 5 ml of wash solution I for every 10 cm^2 membrane). Wash the array by hand rolling the bottles for 5 minutes behind a radiation shield. Decant and discard the wash solution in designated waste container.
g. Repeat step 7 two more times.
h. Add wash Solution I and incubate/wash in hybridization oven at 42°C for 20 min at max rpm.
i. Decant wash solution I and add wash Solution II (below; pre-warmed to 42°C) and incubate/wash in hybridization oven at 42°C for 20 min at max rpm (use at least 5 ml of wash II for every 10 cm^2 membrane).
j. Decant wash solution II and add wash Solution III (pre-warmed to 42°C) and incubate/wash in hybridization oven at 42°C for 20 min at max rpm (use at least 5 ml of wash II for every 10 cm^2 membrane).
k. Decant Wash Solution and use forceps to gently pull out the membrane.
l. Wrap hybridized membranes in Saran wrap. When using Saran wrap, care should be taken to not crease the membrane or wrinkle the Saran wrap. Wrinkles will prevent the hybridized membrane from sitting flat against the phosphoscreen resulting in a blurry image.
m. Expose to phosphor screens for approximately 5 days.
n. Scan phosphoscreen using the storm 840 PhosphoImager (Molecular Dynamics).
o. The fluorescence intensity of each spot will be quantified automatically using the Array-Pro analysis software version 1.0 (Media Cybernetics, Carlsbad, CA).

Prehybridization /Hybridization Solution (makes 100 mL)
50 ml formamide
25 ml 0.5 M sodium phosphate, pH 7.2
15 ml ddH_2O
1.46 g NaCl
7 g SDS
Bring to 100 ml with ddH_2O.
Store at 4°C until used. If precipitation occurs, heat the solution as necessary.

20X SSC
3 M NaCl
0.3 M sodium citrate
Adjust to pH 7.0 with HCl

Wash solution I (2x SSC/0.1% SDS)	**Wash Solution II (0.5x SSC/0.1% SDS)**	**Wash solution III (0.1x SSC/0.1% SDS)**
100 ml 20x SSC	25 ml 20x SSC	5 ml 20x SSC
5 ml 20% SDS	5 ml 20% SDS	5 ml 20% SDS
895 ml ddH_2O	970 ml ddH_2O	990 ml ddH_2O

3 Target synthesis protocols for cDNA and 70mer oligo arrays

The following protocols have been successfully used by us to obtain expression profiles on *M. truncatula* cDNA and 70mer oligonucleotide microarrays. A number of other protocols and variations in target synthesis and probe hybridization exist and should work comparably well.

A critical point is the amount and integrity of the RNA used for target synthesis. In general, we successfully used a range of commercial RNA isolation protocols (e.g. Qiagen RNeasy), including TriReagent® and phenol-based methods. Please note that due to the presence of reverse transcriptase inhibitors in seed coats, particular stages of legume seeds require specific protocols for RNA isolation (e.g. hot-phenol protocols); and also other tissues might require particular isolation protocols or column purification. In case non-column RNA isolation protocols were used, we in general recommend to purify the RNA by either Microcon-30 filters (Millipore) or Qiagen RNeasy cleanup kits. In addition, thorough quality-checks, e.g. using a Bioanalyzer (Agilent), should be common practice.

As a rule of thumb, you should not try to save total RNA as long as there is no limitation to this resource. If the amount of RNA is limited, exponential PCR target amplification, linear T7 target amplification or signal amplification methods, e.g. using dendrimer technology (Genishere), have proven to be useful.

70mer oligonucleotide probes represent the sense strand probe orientation, so make sure to use antisense targets in your experiments, e.g. hexamer- or oligo dT-primed cDNA obtained by reverse transcription, PCR-amplified double-stranded cDNA or amplified antisense-RNA.

Please note that we use the definitions "probe = cDNA- or 70mer oligonucleotide spotted on the slide" and "target = labeled molecules, usually first-strand cDNAs, that are used in hybridizations" throughout the protocols.

3.1 Synthesis of fluorescence-labeled cDNA-targets by reverse transcription

Components stored at -20°C

- 5×RT buffer (Invitrogen, delivered with Superscript II or Superscript III Reverse Transcriptase)
- 50×dNTP stock solution (see below)
- 0.1 M DTT (Invitrogen, delivered with Superscript II or Superscript III Reverse Transcriptase)
- Superscript II or Superscript III Reverse Transcriptase (200 U/µl; Invitrogen)
- RNAse inhibitor (40 U/µl; Invitrogen)
- 0.2 M NaOH and 0.2 M HCl (Merck)
- 4 M hydroxylamine (Sigma, dissolve in MilliQ water)
- 1 M sodium bicarbonate pH9 (Sigma, dissolve in MilliQ water and adjust pH, pH is important)

Components stored at 4°C (-20°C after aliquoting)

- Cy3-NHS ester or Alexa555/Alexa532/Alexa546-NHS ester; Cy5-NHS or Alexa647-NHS ester

- in each case, 1/6th of one aliquot of the monoreactive dye from Amersham is used for one labeling, mg values are not stated by the supplier (estimated between 10 and 50 µg).
- preparing aliquots: dissolve NHS esters in 10 µl of water-free DMSO, it is essential to avoid any contact of the dyes with water prior to labeling. Immediately re-seal DMSO with fresh dessication packs, aliquot 1.5 µl of NHS esters into 10 brown Eppendorf tubes, speed-vac in the dark for 45 min, seal dried NHS esters in plastic bags together with dessication packs, store at -20°C. Take care: some batches of NHS-esters may require to aliqout more than 1.5 µl!

Components stored at RT

- CyScribe GFX columns as well as capture, washing and elution buffer (Amersham Biosciences)
- (alternatively) Microcon-30 filters (Millipore)

You need to supply

- your favoured total RNA prepared by the methods mentioned above
- amino-modified random hexamer primers (dissolved in DEPC-water)
- RNAse-free Eppendorf tubes and tips (e.g. from Peqlab)
- autoclaved MilliQ water
- DEPC-treated water (e.g. from the Qiagen™ RNeasy kit)
- 80% ethanol (diluted to 80% from absolute ethanol, Merck)

Preparations

First, prepare a 100 mM aminoallyl(aa)-dUTP solution as follows:

- dissolve appropriate amounts of aa-dUTP in DEPC-treated H2O and 1 N NaOH to reach a 100 mM solution: dissolve 1 mg in 17 µl DEPC-treated H_2O and 0.68 µl 1 N NaOH OR dissolve 5 mg in 85 µl DEPC-treated H_2O and 3.4 µl 1 N NaOH. Measure pH with a drop on a pH filter paper and adjust pH to 7.0 immediately using 0.1-0.2 µl of 1N NaOH. Store (aa)-dUTP solutions at -20°C.

  Then, prepare a 50×dNTP (2:1 aa-dUTP/dTTP mix) stock as follows, store at –20°C in aliquots.
  - 100 mM dATP (e.g. from Peqlab): 10.0 µl (final concentration 25 mM)
  - 100 mM dCTP (e.g. from Peqlab): 10.0 µl (final concentration 25 mM)
  - 100 mM dGTP (e.g. from Peqlab): 10.0 µl (final concentration 25 mM)
  - 100 mM dTTP (e.g. from Peqlab): 3.3 µl (final concentration 8 mM)
  - 100 mM aa-dUTP (Sigma, Molecular Probes): 6.7 µl (final concentration 17 mM)
  - We recommend the 50×dNTP (2:1 aa-dUTP/dTTP mix) stock for use with AT-rich targets to avoid cross-bleaching of Cy-dyes in AT-rich stretches. To obtain a 50×dNTP (4:1 aa-dUTP/dTTP mix) stock (e.g. for labeling genomic DNA), use 2.0 µl (final concentration 5 mM) 100 mM dTTP and 8.0 µl (final concentration 20 mM) 100 mM aa-dUTP instead.

Protocol for labeling reverse-transcribed total RNA for microarray hybridizations

- preheat the 42°C and 70°C heating blocks 30 min before starting and prepare an ice bucket
- wear gloves. Use filter tips, autoclavable pipetmen and RNAse-free Eppendorf tubes

- thaw DEPC H_20, 5xRT first strand buffer, 0.1 M DTT and primers

Reverse transcription of total RNA to yield aminoallyl-labeled first-strand cDNA

- mix by flicking and spin down:
- 15 to 30 µg of total RNA purified using Microcon-30 filters up to 16.0 µl
- double anchored oligodT_{15}VN primers [2.5 µg/µl] 2.0 µl
- OR amino-modified random hexamers [5 µg/µl] 2.0 µl
- OR a combination of both (we recommend 2 µl of a 1:1 [final: 1.25/2.5 µg/µl] dT_{15}VN/amino-modified random hexamers mix for 70mer oligonucleotide slides)
- add DEPC-treated H2O to 18 µl (duplication of volume is possible until the clean-up step!)

- incubate at 70 °C for 10 min in a heating block
- incubate at 0 °C for 5 min on ice (primer annealing), quickly spin down
- (optional) pre-incubate at 42°C for 2 min in a heating block (NOT for priming that includes hexamers, these otherwise dissociate)

During the 0°C incubation, prepare a nucleotide premix as follows (volumes are for a 1.0× mix; prepare 1.1 × volumes per target) in an RNAse-free Eppendorf tube.

- 5×RT first strand buffer 6.0 µl
- 0.1 M DTT 3.0 µl
- RNase inhibitor [40 U/µl] 0.5 µl
- Superscript II or Superscript III RT [200 U/µl] 1.5 µl
- 50×dNTP stock solution including aa-dUTP 0.6 µl (2:1 aa-dUTP/dTTP nucleotide mix)
- mix by flicking, spin down, leave at RT until use. RNAse Inhibitor, Superscript II/III and 50×dNTP should be added immediately before use (do NOT store this mix on ice)
- at RT, add 11.6 µl of the nucleotide premix to each annealing reaction, mix by flicking, spin down
- incubate at 42 °C for 1 h (Superscript II used) or for 2 h (Superscript III used) in a heating block
- <u>This step is only required if Superscript II is used</u>: add 1 µl of Superscript II [200 U/µl], quickly stirr with the tip, incubate at 42 °C for 1 h in a heating block
- place 0.2 N NaOH, 0.2 N HCl, 1 M sodium bicarbonate and 4 M hydroxylamine at RT
  from now on, RNAse-free conditions are not required
- <u>either (for CyScribe GFX purification)</u>: wearing gloves, place CyScribe GFX columns (one per labeling) in collection tubes and prepare one empty 1.5 ml tube per labeling. Label collection tubes at their side. Do not label the column and make sure during the whole procedure that columns are not mixed up. Label the 1.5 ml tubes at the side and cut off the lid.
- thaw and vortex 1 M sodium bicarbonate, pH 9.0 to dissolve white precipitates
- prepare 0.1 M sodium bicarbonate (pH 9.0) by diluting the 1 M stock solution in MilliQ water, vortex 1 M sodium bicarbonate (pH 9.0) to dissolve white precipitates, 60 µl 0.1 M sodium bicarbonate (pH 9.0) will be required per labeling reaction. Prepare 80% ethanol from absolute ethanol using MilliQ water (1.8 ml per labeling).

- or (for Microcon-30 purification): wearing gloves, place Microcon-30 filters (one per labeling) in collection tubes (red ring up) and prepare 3 empty collection tubes in a row per Microcon-30 filter. Label Microcon-30 at their side. Label the final set of tubes at the side and cut off the lid.
- thaw and vortex 1 M sodium bicarbonate, pH 9.0 to dissolve white precipitates

Hydrolysis of RNA

- add 15 µl of commercial (DO NOT prepare yourself) 0.2 M NaOH (arrested pipetman!) using 100 µl filter tips (exact flowout)
- mix by flicking and spin down
- incubate at 70 °C for 10 min in a heating block
- add 15 µl of commercial (DO NOT prepare yourself) 0.2 M HCl using the arrested pipetman and 100 µl filter tips (exact flowout), mix immediately by pipetting up and down to avoid precipitates
- either (for CyScribe GFX purification): immediately after each target is neutralized (do not neutralize all parallel targets first), quickly proceed with CyScribe GFX column purification
- or (for Microcon-30 purification): immediately after each target is neutralized (do not neutralize all parallel targets first), quickly add 450 µl MilliQ H_2O (quick dilution is essential in case pH is not ok) and pipet up and down two times. In case you combine targets that are to be coupled to the same dye later on at this step, use 390 µl (2 targets) or 330 µl (3 targets) instead. Start to dilute the first target in MilliQ H_2O, then add the other target(s). Vortex all Eppendorf tubes together and spin down. Proceed with Microcon-30 filter purification.

In these and the next steps, do NOT use Tris-containing buffers instead of water, since the amino groups will interfere with the subsequent coupling

Clean-up of aminoallyl-labeled first-strand cDNA

- perform clean-up of aminoallyl-labeled first-strand cDNA using either the following Cyscribe GFX (recommended) or the Microcon-30 protocol (more time-consuming)

Clean-up of aminoallyl-labeled first-strand cDNA (removal of nucleotides and other low-molecular-weight molecules with amino groups) using CyScribe GFX columns

- directly after neutralization of one labeling reaction add 450 µl capture buffer to the reaction and mix by pipetting up and down (proceed with this step until all labeling reactions have been neutralized and mixed with capture buffer), samples should not stay in capture buffer longer than 5 min
- add the complete neutralized mix to a CyScribe GFX column
- spin at 13.000 rpm for 30 sec at 20°C in a microcentrifuge and discard flowthrough
- add 600 µl of 80% ethanol (it is very important not to use less concentrated ethanol, do not use the washing buffer provided with the CyScribe GFX purification kit)
- spin at 13.000 rpm for 30 sec at 20°C in a microcentrifuge and discard flowthrough
- repeat this washing step twice

- spin at 13.000 rpm for 10 sec at 20°C in a microcentrifuge and place column in a new 1.5 ml tube
- add 60 µl 0.1 M sodium bicarbonate (pH 9.0), do not use the elution buffer provided with the CyScribe GFX purification kit
- incubate for 5 min at room temperature
- spin at 13.000 rpm for 1 min at 20°C in a microcentrifuge
- storage of first strand cDNA eluted from the column in 0.1 M sodium bicarbonate at -20°C is not recommended by the manufacturers of aminoallyl fluorescent labeling kits, manufacturers recommend to proceed immediately with the coupling of fluorescent dyes (below), we have not observed a negative effect of storage of first strand cDNA in 0.1 M sodium bicarbonate at -20°C overnight

ALTERNATIVE clean-up of aminoallyl-labeled first-strand cDNA (removal of nucleotides and other low-molecular-weight molecules with amino groups) using Microcon-30 filters

- add the complete neutralized mix to a Microcon-30 filter
- spin at 12.000 rpm for 10 min at 20°C in the Jouan BR4i centrifuge (other centrifuges: test different times, e.g. 8 min in case of the Heraeus Biofuge pico) and discard flowthrough
- place filter in new collection tube
- add 450 µl of MilliQ H_2O
- spin at 12.000 rpm for 10 min at 20°C in the Jouan BR4i centrifuge (other centrifuges: test different times, e.g. 8 min in case of the Heraeus Biofuge pico) and discard flowthrough
- place filter in new collection tube
- add 450 µl of MilliQ H_2O
- spin at 12.000 rpm for 9 min at 20°C in the Jouan BR4i centrifuge (other centrifuges: test different times, e.g. 8 min in case of the Heraeus Biofuge pico) and discard flowthrough
- check that at least 10 µl and at most 20 µl are left on top of the membrane within the red ring; if necessary, spin another 15-30 sec at 12.000 rpm at 20°C in the Jouan BR4i centrifuge (other centrifuges: test different times, e.g. 5-15 sec in case of the Heraeus Biofuge pico) and discard flowthrough
- place Microcon-30 filter upside-down in a new collection tube (cut off cover lid!) and spin for 1 min at 13.000 rpm
- now, at least 10 µl should be in the collection tube. If not, invert the Microcon-30 filter and add 10 µl to the membrane. Leave it for 1 min at RT to completely elute cDNA from the filter. Then, place the Microcon-30 filter upside-down in the same collection tube and spin for 1 min at 13.000 rpm
- estimate the eluted volume using a fine 100 µl filter tip and add the approximately necessary volume of MilliQ H_2O to obtain 20 µl (e.g. by soaking up the remaining volume of MilliQ H_2O from a microplate).
- in case you end up with more than 20 µl (maximum 30 µl), it is essential to adjust the amount of 1 M sodium bicarbonate pH 9.0 that is added in the next step accordingly!
- transfer samples to new Eppendorf tubes, these samples can be stored at -20 °C
- vortex 1 M sodium bicarbonate, pH 9.0 to dissolve white precipitates

- add 1 µl (or proportionally more, if more than 20 µl were eluted from the Microcon-30 filter) of 1 M sodium bicarbonate, pH 9.0 to 20 µl of aminoallyl labeled first strand cDNA (final concentration 50 mM), mix by flicking and spin down
- storage of first strand cDNA in sodium bicarbonate at -20°C is not recommended by the manufacturers of aminoallyl fluorescent labeling kits, manufacturers recommend to proceed immediately with the coupling of fluorescent dyes (below), we have never tried storage of first strand cDNA in sodium bicarbonate at -20°C and therefore cannot give any recommendations

Coupling of fluorescent dyes to the aminoallyl-labeled first-strand cDNA

- protect samples from light all the time using brown Eppendorf tubes, avoid room light and direct sunlight
- dissolve Cy3- or Cy5-NHS or Alexa-NHS esters provided in aliquoted form (see above) in brown Eppendorf tubes in the complete aa-containing first strand cDNA by pipetting up and down several times until the dye is dissolved (red/blue colour!)
  alternatively, 1.5 µl of fluorescent dye in DMSO (orginal dye pack aliquot from Amersham dissolved in 10 µl DMSO, cannot be stored, always prepare fresh!) can be added to the aa-containing first strand cDNA solution
- from now on, work in brown Eppendorf tubes to protect the fluorescent dyes
- do NOT spin down, just tap down drops from the side of the Eppendorf tubes
- incubate for 1 h at RT in the dark (up to 2 h is possible)

Quenching (blocking of all remaining dyes with the amino groups from the hydroxylamine)

- add 4.5 µl of 4 M hydroxylamine
- mix by flicking, do NOT spin down
- leave for 15 min at RT in the dark

Clean-up of fluorescently labeled targets using CyScribe GFX Purification Kit (Amersham Biosciences)

Cy5- and Cy3-labeled targets to be hybridized simultanuously to one microarray are cleaned up together. In the event that only one of either dye is used for hybridization or has to be purified for other purposes, use the same volumes as specified below.

- work quickly to protect labeled targets from the light, Cy5 bleaches quickly and is particularly sensitive to high ozone concentrations!
- add 600 µl capture buffer (CyScribe GFX Purification Kit, Amersham Biosciences) to the Cy5-labeled sample and mix by pipetting up and down, then add the Cy3-labeled sample to this solution and mix by pipetting up and down
- apply all to a GFX column in a collection tube (CyScribe GFX Purification Kit), do not leave the sample in capture buffer for more than 5 min
- spin at full speed (appr. 10.000-13.000 rpm) for 30 sec
- discard flowthrough
- add 600 µl washing buffer (CyScribe GFX Purification Kit)

- spin at full speed (appr. 10.000-13.000 rpm) for 30 sec
- discard flowthrough
- add 600 µl washing buffer (CyScribe GFX Purification Kit)
- spin at full speed (appr. 10.000-13.000 rpm) for 30 sec
- discard flowthrough
- add 600 µl washing buffer (CyScribe GFX Purification Kit)
- spin at full speed (appr. 10.000-13.000 rpm) for 30 sec
- discard flowthrough
- spin at full speed (appr. 10.000-13.000 rpm) for 10 sec
- transfer the dried GFX column to a fresh brown Eppendorf tube labeled at the side, since the cover lid has to be cut off
- add 60 µl elution buffer (CyScribe GFX Purification Kit) to the center of the filter
- leave for 5 min at RT
- spin at full speed (appr. 10.000-13.000 rpm) for 1 min
- the resulting 60 µl of combined Cy3/Cy5-labeled targets are transferred to a fresh brown tube with a screw cap. Label on the sides and seal with "Tesafilm".
- remove an aliquot for checking the efficiency of target synthesis
- freeze this aliquot as well as the Cy-labeled target at -20 °C until use

3.2 Synthesis of fluorescence-labeled cDNA targets by PCR-amplification

First strand cDNA synthesis from total RNA

- combine the following reagents in a sterile RNAse-free 0.5-ml reaction tube (5 µl total volume):
  - 1-3 µl RNA sample (0.05-1 µg of total RNA)
  - 1 µl cDNA synthesis (CDS) primer (10 µM, SMART Synthesis Kit, Clontech)
  - 1 µl SMART II oligonucleotide (10 µM, SMART Synthesis Kit, Clontech)
  - x µl DEPC water
- mix content and spin the tube briefly in a centrifuge
- incubate the tube at 70°C in a thermal cycler for 2 min, briefly spin down and keep tube at RT
- add the following to each reaction tube:
  - 1 µl Dithiothreitol (20 mM)
  - 1 µl dNTP-mix (10 µM)
  - 2 µl 5×first-strand buffer
  - 1 µl SuperScript II Reverse Transcriptase (Invitrogen)
- gently vortex, briefly spin down and incubate at 42°C for 1 hour in a thermal cycler
- add 40 µl TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA) and heat tubes at 70°C for 7 min
- mix in a PCR-tube or PCR-strip (for one amplification):
  - 38.3-39.3 µl H_2O
  - 5 µl Advantage buffer (Advantage PCR Kit, Clontech)
  - 0.7 µl 50x dNTP stock solution including aminoallyl-dUTP (2:1 aadUTP/dTTP mix) (see: Synthesis of fluorescence-labeled cDNA-targets by reverse transcription)
  - 2 µl SMART PCR-primers (10 µM, SMART Synthesis Kit, Clontech)
  - 1 µl Advantage Polymerase-Mix (Advantage PCR Kit, Clontech)
  - 1-2 µl first strand cDNA

- to determine optimal cycle numbers required for amplification, perform alternative PCRs using the following programmes for a thermal cycler with a heated cover (e.g. MJ Research)
    - 95°C 1 min
    - amplify for 12,14,16,18 and 20 cycles of [94°C 15 sec; 65°C 30 sec; 68°C 6 min]
    - 68°C 6 min
    - cool to 4°C and freeze targets until use at -20°C
- check 5 µl of each amplification reaction on a 0.8 %(w/v) agarose gel, a smear should be visible. If not, optimize the PCR-amplifications using dNTP mixes without aminoallyl-dUTP! Use the amplification reaction giving the first visible smear. This minimizes artefacts arising from unequal PCR-amplifications of different targets and reduces a plateau effect resulting from overcycling. The plateau effect results in the unproportional amplification of low-abundance transcripts. Alternative to a direct incorporation of aminoallyl-dUTP, PCR-amplification can be performed with dNTP mixes without aminoallyl-dUTP. PCR-products can subsequently be labeled with aminoallyl-dUTP using amino-modified random hexamers and aminoallyl-dUTP containing nucleotide mixes.

Clean-up of aminoallyl-containing PCR-products using CyScribe GFX columns and Cy-labeling

- wearing gloves, place CyScribe GFX columns (one per PCR) in collection tubes and prepare one 1.5 ml tube per labeling. Do not label the column and make sure that columns are not mixed up. Label the 1.5 ml tubes at the side and cut off the lid.
- prepare 0.1 M sodium bicarbonate (pH 9.0) by diluting the 1 M sodium bicarbonate (pH 9.0) stock solution in MilliQ water, vortex to dissolve white precipitates; 60 µl 0.1 M sodium bicarbonate (pH 9.0) will be required per labeling. Prepare 80% ethanol from absolute ethanol using MilliQ water.
- add 450 µl capture buffer to the remaining 45 µl of aminoallyl-dUTP containing PCR product, mix by pipetting up and down and continue with the cleanup protocol (do not leave the aminoallyl-dUTP containing PCR products longer than 5 min in capture buffer) as described for labeling targets by reverse-transcription
- after elution using in 0.1M sodium bicarbonate, denature double-stranded targets for 1 min in a boiling water bath, cool on ice/water and spin down
- continue with the section *Coupling of fluorescent dyes to the aminoallyl-labeled first-strand cDNA* of the "Synthesis of fluorescence-labeled cDNA-targets by reverse transcription", follow it to the end.

3.3 Synthesis of fluorescence-labeled aRNA targets by 1-step T7-amplification

- wear gloves, use filter tips, autoclavable pipetmen, and RNAse-free Eppendorf tubes, since the amplified hybridization targets are RNA molecules
- kits and components used: TargetAmp™ 1-Round Aminoallyl-aRNA Amplification Kit 101 (EpiCentre) supplemented with Superscript III (Invitrogen), RNeasy MinElute Cleanup Kit (Qiagen) or RNeasy Mini Kit (Qiagen), aliquoted Cy-dyes (Amersham)
- check RNA purity on Nanodrop or Agilent Bioanalyzer prior to T7 amplification.
- please do also consult the relevant manuals of the kit providers mentioned below
- thaw EpiCentre kit components immediately before use and quickly freeze them again
- prepare an ice bucket, 37°C, 42°C, 50°C, 65°C and 80°C heating blocks/water baths

First-strand cDNA synthesis (per reaction)

- maximum probe volume is 2 µl. This volume should contain no more than 500 ng of total RNA. We have tested the procedure down to 100 ng of total RNA with no apparent loss of quality.
- anneal the TargetAmp T7-Oligo(dT) Primer A to the total RNA
  x µl RNase-free water
  x µl total RNA sample
  1 µl TargetAmp T7-Oligo(dT) Primer A (Epicentre)
  3 µl total reaction volume
- incubate at 65°C for 5 minutes
- chill on ice for 1 minute
- quick spin the sample
- add 2 µl of the following 1st-Strand cDNA Synthesis Master Mix per reaction
  1.50 µl TargetAmp Reverse Transcription PreMix-SS (Epicentre)
  0.25 µl DTT (Epicentre kit, NOT the one provided with the SuperScript III)
  0.25 µl SuperScript III Reverse Transcriptase (Invitrogen, 200 U/ml)
  2 µl total (GENTLY mix MasterMix)
- GENTLY mix the reaction
- incubate at 50°C for 30 minutes

Second-strand cDNA synthesis (per reaction)

- add 5 µl of the following 2nd-Strand cDNA Synthesis Master Mix per reaction
  4.5 µl TargetAmp DNA Polymerase PreMix-SS 1 (Epicentre)
  0.5 µl TargetAmp DNA Polymerase-SS 1 (Epicentre)
  5.0 µl total (GENTLY mix MasterMix)
- GENTLY mix the reaction
- incubate at 65°C for 10 minutes
- quick spin the sample
- incubate at 80°C for 3 minutes
- quick spin the sample
- chill on ice. Store at –20°C if necessary or proceed immediately.

in vitro transcription of aminoallyl-containing aRNA (per reaction)

- add 40 µl of the following In Vitro Transcription Master Mix per reaction
  9.6 µl RNase-free water
  4.0 µl TargetAmp T7 Transcription Buffer (Epicentre, thaw at RT. Heat to 37°C if precipitates are visible, mix THOROUGHLY and keep at RT until use)
  16 µl TargetAmp In Vitro Transcription PreMix B (Epicentre)
  2.4 µl aminoallyl-UTP (Epicentre)
  4.0 µl DTT (Epicentre kit, NOT the one provided with the SuperScript III)
  4.0 µl TargetAmp T7 RNA Polymerase (Epicentre, keep on ice!)
  40 µl total (GENTLY mix MasterMix)
- GENTLY mix the reaction and incubate at 42°C EXACTLY for 4 hours
- add 2 µl of RNase-Free DNase I (Epicentre), mix GENTLY and incubate at 37°C for 15 minutes

Aminoallyl-aRNA purification

- the purification column to use is dependent upon the expected yield of aminoallyl-aRNA (AA-aRNA). Use the table on page 8 of the EpiCentre manual to estimate the yield of AA-aRNA expected from the amount of total RNA used in each amplification reaction.
- if the expected yield of AA-aRNA is < 40 µg (typically for less than 250 ng total RNA): purify the AA-aRNA using the RNeasy MinElute Cleanup Kit (Qiagen)
- if the expected yield of AA-aRNA is > 40 µg (typically for more than 250 ng total RNA): purify the AA-aRNA using the RNeasy Mini Kit (Qiagen)
- use the RNase-free water provided in the MinElute Cleanup Kit or the RNeasy Mini Kit. The following procedure should be used with either the MinElute Cleanup Kit or the RNeasy Mini Kit.
- prepare 350 µl of RLT/β-ME solution for each sample. Combine the RLT/β-ME in the ratio of 1 ml of RLT (provided in the purification kits) with 10 µl of β-ME (β-mercaptoethanol) as described in the purification kit manual
- prepare 650 µl of RPE solution for each sample by diluting 1 volume of Buffer RPE (provided in the purification kits) with 4 volumes of 96-100% ethanol as described in the purification kit manual
- to each sample add
  48 µl RNase-free water
  350 µl RLT/β-ME solution
  250 µl 100% ethanol
- apply each sample to the purification kit's spin column in a 2 ml collection tube. Centrifuge at >10.000 rpm for 15 seconds. Discard the flow-through
- apply 650 µl RPE solution onto the column. Centrifuge at >10.000 rpm for 15 seconds. Discard the flow-through.
- apply 650 µl 80% ethanol onto the column. Centrifuge at >10.000 rpm for 15 seconds. Discard the flow-through.
- transfer the spin column into a new collection tube. Open the cap and centrifuge at full speed for 5 minutes.
- transfer the spin column to a 1.5 ml collection tube
- elute the aminoallyl-aRNA:
- if using the MinElute Cleanup Column, apply 15 µl of RNase-free water directly onto the center of the silica-gel membrane. Wait for 5 minutes. Centrifuge at full speed for 1 minute
- if using the RNeasy Mini Column, apply 50 µl of RNase-free water directly onto the center of the silica-gel membrane. Wait for 5 minutes. Centrifuge at full speed for 1 minute
- store the purified aminoallyl-aRNA at –80°C until use

Quantifying concentration, yield, fold-amplification and size range of the aminoallyl-aRNA

- if desired, proceed as described in the Epicentre T7 amplification kit manual. The aRNA concentration can be checked using the Nanodrop, the aRNA size range either on an agarose gel or on the BioAnalyzer. Use 1/30-1/15 vol of purified aminoallyl-aRNA for this purpose. Typically, 10-40 µg of aRNA can be obtained from 100-500 ng of total RNA.

Coupling of Cy-NHS esters the aminoallyl-aRNA

The aminoallyl-aRNA produced can be readily labeled with amine-reactive N-hydroxysuccinimide (NHS) ester of fluorescent dyes (Cy™3-NHS or Cy™5-NHS; Amersham). In aqueous solution, the -NHS group rapidly and with high efficiency reacts with the amine groups of the aminoallyl-uridine incorporated into the aminoallyl-aRNA during the *in vitro* transcription reaction. The end product is aRNA with covalently attached fluorescent molecules.

- protect samples from light at all times in amber Eppendorf tubes, avoid room light and direct sunlight
- thaw 1 M sodium bicarbonate pH 9 to RT and vortex in case white precipitates form!
- preparing Cy3-NHS or Cy5-NHS esters: in each case, $1/6^{th}$ of one aliquot of the monoreactive dyes from Amersham (PA25001 or PA23001) is used for one labeling. Take care to apply RNAse-free conditions when preparing the aliquots. Dissolve NHS-esters in 10 µl of water-free DMSO (Clontech), it is essential to avoid any contact of the dyes with water prior to labeling. Immediately re-seal DMSO with fresh dessication packs, aliquot 1.5 µl of NHS-esters into 6 brown Eppendorf tubes. Speed-vac in the dark for 45 min, seal dried NHS esters in plastic bags with dessication packs, store at -20°C. Take care: some batches of NHS-esters may require to aliqout more than 1.5 µl!
- adjust the pH of the aRNA produced using 1 M sodium bicarbonate pH 9 (Sigma, dissolve in MilliQ water and adjust pH, the correct pH is important. At non-alkaline conditions the coupling doesnt work efficiently and too high pH values degrade the aRNA).
- add either 1.67 or 5.67 µl 1 M sodium bicarbonate pH 9 to 15 or 50 µl purified aRNA, respectively, mix gently to arrive at a final concentration of 0.1 M sodium bicarbonate pH 9
- dissolve the Cy3- or Cy5-NHS esters provided in aliquoted form (see above) in amber Eppendorf tubes in the complete aminoallyl aRNA by pipetting up and down several times until the dye is dissolved (red/blue colour!)
- alternatively, 1.67 µl of fluorescent dye in DMSO (orgininal dye aliquot from Amersham dissolved in 10 µl water-free (!) DMSO, cannot be stored, always prepare fresh!) can be directly added to the aminoallyl aRNA
- from now on, work in amber Eppendorf tubes to protect the fluorescent dyes
- do not spin down, just tap down drops from the side of the Eppendorf tubes
- incubate for 15 min at RT in the dark to prevent aRNA degradation over time
- as long as the Cy-labeled targets are purified seperately, quenching (10 min in the dark) with 4.5 µl of 4 M hydroxylamine is not required. Quenching is necessary if co-purification is desired.

Purification of Cy-coupled aRNA using the RNeasy MinElute Cleanup Kit or the RNeasy Mini kit

- select the appropriate purification kit based on the expected yield as mentioned above under "Aminoallyl-aRNA purification". Note that the amount of Cy-labeled aRNA will usually be up to 30% lower than the amount of aRNA, so usually the RNeasy MinElute Cleanup Kit will be appropriate.
- purify labeled targets seperately or make sure to quench uncoupled amine groups
- proceed as described above under "Aminoallyl-aRNA purification"
- apply 15 µl or 50 µl of RNase-free water directly onto the center of the silica-gel membrane. Wait for 5 minutes, then centrifuge at full speed for 1 minute.
- remove an aliquot for checking the efficiency of target synthesis
- store Cy-coupled aRNA at –80°C until use

3.4 Analysing the efficiency of target synthesis

Checking Cy-coupled cDNA and aRNA targets

- check 1/30 vol of Cy-labeled cDNA or aRNA targets on 1% (w/v) agarose gels in TA buffer. Run for 20 min at 80 V and check fluorescence incorporation and the size distribution of Cy-targets e.g. on a Typhoon Imager (Amersham). Alternatively, check 1/60 vol on a Bioanalyzer (Agilent).
- check 1/60 vol of Cy-labeled cDNA or aRNA targets on the ND-1000 Spectrophotometer (NanoDrop). Typical values are in the range of 4-8 µg cDNA (total amount obtained by reverse-transcription from 15-30 µg of total RNA) with 0.03-0.06 pg Cy3 or Cy5 per ng cDNA or 10-30 µg of Cy-labeled aRNA (total amount obtained by 1-step T7 amplification from 100-500 ng of total RNA) with 0.02-0.1 pg Cy-label per ng aRNA.

Combining Cy-targets prior to hybridization

- combine similar amounts of Cy-labeled cDNA- or aRNA-samples that will be co-hybridized on one microarray. Usually, 4-8 µg cDNA with 0.03-0.06 pg Cy-label/ng cDNA or 2-5 µg of aRNA with 0.3-0.5 pmol Cy-label/ng aRNA will be sufficient, but up to 20 µg Cy-labeled cDNA/aRNA can be used.

4. Hybridization protocols for cDNA and 70mer oligo arrays

4.1 Processing of Nexterion Slide E microarrays

Mt16kOLI1/Mt16kOLI1Plus 70mer oligonucleotide microarrays (Hohnjec et al. 2005) spotted on Nexterion Slide E (Schott) epoxy surfaces have to be processed prior to hybridizations to block free epoxy groups. To process Nexterion Slide E microarrays, prepare the following solutions immediately before use.

Rinsing solution 1: 250 ml MilliQ H_2O
+250 µl Triton X100
dissolve at 80 °C for 5 min
cool down to room temperature

Rinsing solution 2: 500 ml MilliQ H_2O
+ 50 µl 32 % HCl

Rinsing solution 3: 225 ml MilliQ H_2O
+ 25 ml 1 M KCl

Nexterion BlockE 4x Blocking solution (Schott):
150 ml MilliQ H_2O (15 ml in case only 1-2 slides are processed)
+ 47 µl 32 % HCl (4.7 µl in case only 1-2 slides are processed)
+ 50 ml 4×Blocking solution (5 ml in case only 1-2 slides are processed)

Pre-warm the MilliQ/HCL mix to 50°C, add the 4xBlocking solution (stored at RT) 5 min before use and pre-warm the complete Blocking solution to 50°C for at least 5 min. It is important that

the temperature of the Blocking solution is 50°C at the beginning of the 15 min blocking step. Please note that the Blocking solution is instable and MUST NOT be stored longer!

MilliQ H_2O 500 ml

- take out a sealed package of slides and warm to RT for 5 min
- wearing gloves, take out the desired slides (only touch it at the corner where the scratch is or at the code no. at the bottom, the DNA side faces up when you can read the number or the scratch is at the bottom right corner)
- seal the remaining slides of the box together with a new dessication pack and return the sealed package to 18-20°C, place the old dessication pack in the recycling box

Nexterion Slide E slide processing

- place the slides in a plastic rack and carry out the processing by transferring the racks from one container to the other, occasionally lift the rack up and down during washing
- wash slides for 5 min at room temperature in 250 ml of rinsing solution 1
- wash slides for 2 min at room temperature in 250 ml of rinsing solution 2, repeat this step
- wash slides for 10 min at room temperature in 250 ml of rinsing solution 3
- wash slides for 1 min at room temperature in 250 ml of MilliQ H2O
- incubate slides for 15 min at 50 °C in 200 ml prewarmed blocking solution in a glass container, shake at least every 5 min or apply constant shaking. Use a flat bottom glass container to process 1-2 slides (20 ml of blocking solution) and a multiple glass container to process multiple slides (200 ml blocking solution)
- wash slides for 1 min at room temperature in 250 ml of MilliQ H2O
- place rack on an 12x8 cm plastic microplate cover (Genomics Solutions) containing 2 Kim-wipes and immediately spin in the microplate centrifuge at 1.200 rpm for 3 min. Use a stack of 3 used glass slides at every side of the plastic dish to lift up the rack with the slides, this avoids precipitation artetacts at the side of the slide. Be sure to counter-balance using an appropriate balance

4.2 Hybridization using a cover slip and a slide hybridization chamber

- preheat a 42°C and a 65°C water bath
- dry 60 µl of combined Cy3/Cy5-labeled targets in a speed vac for ~ 30 min until all liquid has evaporated. To protect fluorescent dyes cover the transparent lid of the speed vac.
- during slide processing, prewarm the Telechem hybridization chamber on the lid of the 42°C water bath or on a heated 42°C surface. It is VERY IMPORTANT that this chamber is AT 42°C THROUGHOUT THE WHOLE PROCESS UNTIL THE COVER SLIP IS PLACED to avoid the local precipitation of targets or gradients
- dissolve the completely dried target in 60 µl of DIG Easy Hyb by pipetting up and down in the brown Eppendorf tube. If necessary, combine different Eppendorf tubes at this stage
- add 1 µl of sonicated salmon sperm DNA (5µg/µl)
- the total amount of hybridization solution containing mixed Cy3/Cy5-labeled targets is now 61 µl, incubate at RT in the dark until use

- incubate the hybridization solution containing the mixed Cy3/Cy5-labeled targets in a 65°C waterbath for 5-10 min
- place 7 µl of demineralized water from the tap in each hole at the side of the bottom of the slide chamber. The slide chamber is on top of the water bath or otherwise preheated to 42°C
- place the dried slide with the DNA side facing up into the slide chamber
- quickly spin down the denatured target and apply 58 µl as a drop to the center of the slide without touching the surface
- as quick as possible (!!): take a (clean!) cover slip (22x60mm) and place it on the left side of the slide (4 mm from the top of the slide), the scratch or the slide number is on the right side of the slide. At the right side, hold the cover slip with another cover slip and quickly but carefully lower the cover slip. As soon as it touches the drop, take care that it does not reach over the corners of the slide and lower the cover slip. Avoid swimming of the cover slip from its destined position. A correction of the position of a wrongly placed cover slip is NOT possible since this causes severe problems later. All manipulations are carried out on the lid of the 42°C water bath
- place lid of the slide chamber on the chamber and tighten the four srews carefully but quickly in a diagonal manner
- now, a black ring without interrupts has to be visible surrounding the slide. Then the slide chamber is sealed. Do NOT tighten the screws of the chamber more than necessary, since the plexi glass lid may break!
- place chamber on the bottom of the 42°C water bath. Check that the chamber became humid in the inside (the 14 µl water evaporate now), after a couple of minutes, the atmosphere becomes saturated and the slide is visible again
- hybridize not less than 14 and not more than18 hours at 42°C
- prepare 500 ml each of all washing buffers in demineralized water from appropriate stocks (20×SSC, 10 %(w/v) SDS) and preheat 2×SSC, 0.2 %(w/v) SDS washing buffer to 42°C and cool the final 0.1×SSC (for cDNA targets; use 0.05×SSC for aRNA targets or for higher stringency) washing buffer to 18 °C
- just before hybridization is finished, prepare 2 black plastic boxes each with 250 ml 2×SSC, 0.2 % (w/v) SDS washing buffer prewarmed to 42°C. Also prepare an 12x8 cm petri dish (Genomics Solutions) containing 1 Kim-wipe and stacks of three used slides each at the sides
- hold the chamber horizontally, dry it from the outside and open the screws at diagonal ends
- wearing gloves, remove the slide with the cover slip and quickly dump it while holding it with the fingers into the first container thereby washing off the cover slip
- place the slide in the slide rack and place it in the second black plastic box with 2×SSC, 0.2 %(w/v) SDS washing buffer prewarmed to 42°C. Shake for 5 min. From now on ocasionally move slide racks up and down to avoid air bubbles
- transfer to 0.2×SSC, 0.1 %(w/v) SDS (RT, at most 24°C) in a black plastic rack, shake for 1 min
- transfer to 0.2×SSC, 0.1 %(w/v) SDS (RT, at most 24°C) in a black plastic rack, shake for 1 min
- transfer to 0.2×SSC (RT, at most 24°C) in a black plastic rack, shake for 1 min
- transfer to 0.2×SSC (RT, at most 24°C) in a black plastic rack, shake for 1 min
- transfer to 0.1×SSC or 0.05×SSC (Important: set to 18 °C and remove from the cooler immediately before use) in a black plastic rack, shake for 1 min

- place rack on an 12x8 cm plastic microplate cover (Genomics Solutions) containing 2 Kim-wipes and immediately centrifuge in the microplate centrifuge at 1.200 rpm for 3 min. Use a stack of 3 used glass slides at every side of the plastic dish to lift up the rack with the slides, this avoids precipitation artetacts at the side of the slide. Be sure to counter-balance using an appropriate balance. If necessary, dry corners of the slide afterwards with a Kim-wipe
- place dried slide in a box in the dark until scanning. Work quickly at all times, avoid direct light and exposure to high ozone concentrations, since this strongly enhances Cy5-bleaching.

4.3 Hybridization using the Tecan HS4800 station

- start the HS4800 Control Manager Software
- if a dialog box for setting up a new HS Control Manger license appears click on “Cancel”
- a login window appears
- enter login and password
- switch on the hybridization machine
- insert tubings into the washing solutions
- insert slide adapters with dummy slides into the hybridization modules to be used (always insert 4 dummy slides per module)
- check if screws closing the injection ports are really tightend
- connect the instrument to the computer
- switch on the heating of the Liquid Distribution Unit
- open an appropriate hybridization program
- never change programs yourself, ask experienced personnel
- select the hybridization chambers to be used by clicking on
- prime the channels to be used
- select the channels to be used and prime each for 30 sec (channels can only be primed separately)
- insert slide adapters with microarrays into the hybridization modules (always insert 4 slides per module, if you have less microarrays use additional dummy slides)
- start the program
- remove the Cy3/5 labeled combined targets from the refrigerator (they should be in brown Eppendorf tubes with a screw cap) and speed-vac them for appr. 15 min until the volume is reduced to not more than 10 µl. Add DIG Easy Hyb hybridization solution containing 1.5 µl of salmon sperm DNA (5 µg/µl) to a final volume of 100 µl and mix by pipetting up and down. Spin down in a centrifuge at 13.000 rpm and keep in the dark.
- incubate at 65°C for 5 min
- wait until a notification window “Preparing probe injection” appears (an acustic signal reminds you of injecting the samples)
- press OK on the control panel of the instrument
- open the injection port of the first hybridization chamber to be used
- inject the first sample (100 µl) using a pipetman (do NOT use filter tips)
- close the injection port
- press OK on the control panel of the instrument
- open the injection port of the second hybridization chamber to be used
- inject the second sample, press OK on the control panel of the instrument

- close the injection port
- proceed with the remaining samples following the same procedure until all samples have been injected
- after the last confirmation of injection by pressing OK the program continues automatically with the hybridization step
- prepare 250 ml or 500 ml, respectively (250 ml are needed for each individual washing step listed below), of the washing buffers in demineralized water from appropriate stocks (20×SSC, 10 %(w/v) SDS), preheat 2×SSC, 0.2 %(w/v) SDS washing buffer to 42°C and cool the 0.1×SSC (for cDNA targets; use 0.05×SSC for aRNA targets or higher stringency) washing buffer to 18 °C
- prepare 2 black plastic boxes and one black plastic slide rack. Also prepare an 12x8 cm petri dish (Genomics Solutions) containing 1 Kim-wipe and stacks of three used slides each at the sides
- just before the program terminates, pour 250 ml 2×SSC, 0.2 %(w/v) SDS washing buffer prewarmed to 42°C in 1 black plastic box
- wearing gloves, remove the slide adapters one by one from the hybridization modules (do not touch the DNA side of the slide, only touch the edges of the slide!), quickly remove the slides one by one from the adapter and place them into the black plastic slide rack that is immersed in the first container in the prewarmed 2×SSC, 0.2 %(w/v) SDS washing buffer
- move up and down several times immediately and shake for 1 min on a horizontal shaker after removal of the last slide. From now on regularly move slide racks up and down to avoid air bubble formation
- proceed with the following washing steps:
  - transfer to 0.2×SSC, 0.1 %(w/v) SDS (RT, at most 24°C) in a black plastic rack, shake for 1 min
  - transfer to 0.2×SSC, 0.1 %(w/v) SDS (RT, at most 24°C) in a black plastic rack, shake for 1 min
  - transfer to 0.2×SSC (RT, at most 24°C) in a black plastic rack, shake for 1 min
  - transfer to 0.2×SSC (RT, at most 24°C) in a black plastic rack, shake for 1 min
  - transfer to 0.1×SSC or 0.05×SSC (Important: set to 18 °C and remove from the cooler immediately before use) in a black plastic rack, shake for 1 min
- place rack on an 12x8 cm plastic microplate cover (Genomics Solutions) containing 2 Kim-wipes and immediately centrifuge in the microplate centrifuge at 1.200 rpm for 3-5 min. Use a stack of 3 used glass slides at every side of the plastic dish to lift up the rack with the slides, this avoids precipitation artetacts at the side of the slide. Be sure to counter-balance using an appropriate balance. If necessary, dry corners of the slide afterwards with a Kim-wipe
- place dried slides in a box in the dark until scanning. This may avoid bleaching effects in the Cy5 channel. Work quickly at all times, avoid direct light and exposure to high ozone concentrations, since this strongly enhances Cy5-bleaching.
- insert slide adapters with dummy slides in all modules that were used (always insert 4 slides per module, even if less than 4 slides have been used for hybridization)
- switch off the heating of the Liquid Distribution Unit
- insert tubings of all channels that have been used into a 2 l bucket filled with filtered and autoclaved MilliQ wate
- rinse the channels that have been used

- only single selected channels can be rinsed at once or all 6 channels of the instrument can be rinsed in parallel
- remove all tubings from the solutions
- open the nitrogen gas supply
- open the program “Final drying” and select all slides of each module that has been used for hybridization
- start the program
- after the drying program has finished remove the adapters from all modules that have been used
- clean the chambers and injection ports with demineralized water and dry the chambers using Kim wipes
- close the nitrogen gas supply
- disconnect the instrument by clicking on
- close the HS4800 Control Manger Software
- switch off the HS4800 hybridization station

5 Scanning of hybridized microarrays using the Tecan LS Reloaded Scanner

- switch on the computer and log in
- switch on the Tecan LS Reloaded Microarray Scanner
- double click on the icon to start the Array-Pro Analyzer software
- click on “Aquire” in the menu and select the “Tecan Scanner”
- a dialog box appears
- click on OK
- do NOT change the scanner or stacker ports
- the “Measurement Control” dialog box appears
- click on “Laser Control”, a dialog box appears, check the boxes for the required laser(s), prewarming of the lasers requires at least 30 min
- select the appropriate template file specifying scanning parameters by clicking on “Load”
- define these parameters according to the microarray surface used, the microarray glass type (important to set autofocus paramaters). Enquire with your local facility for optimal parameter setting. Usually, the Automatic Gain Control (AGC) mode should be used to maximize signal range and minimize signals in saturation.
- if it is required to change the photomultiplier (PMT) settings for the Cy5 and Cy3 channels and/or the oversampling factor click on edit, a dialog box appears, choose the card “Scan Settings” and change the parameters, do NOT change the pinhole settings
- if you just want do scan one channel, choose the card “General” and select the Scan Mode “Single”, do NOT use the Scan Mode “Dual parallel” (this reduces the dynamic range, and the scanner is not calibrated for this mode)
- do NOT change any other settings and do NOT overwrite the template file
- click OK after the required changes were made
- choose the number of slides to be scanned by clicking on the green (activated) or grey (inactivated) areas of the symbolized slide adapter

- under “Settings” click on “Slide Settings”, a dialog box appears, enter appropriate names for your microarrays and activate the correct orientation of your slides in the adapter, do NOT uncheck the “AutoSave” button
- depending on the scanner settings the DNA side has to face up or down in the slide adapter. The barcode can only be read by the instrument if it is on the side of the adapter opposite to the tongue and if the barcode faces up
- click OK
- to load up the slide adapter click on “Plate/Filter” in the “Measurement control” window, a dialog box appears
- to open the scanner lid click on Plate: “Out”
- place the adapter with the tongues directed to the front of the instrument in the scanner
- to close the scanner lid click on Plate: ”In”
- do NOT click on Filter slide 1: Out OR Filter slide 2: Out
- you may now close the “Movements” dialog box
- to start the scanning process click on “Start” in the “Measurement control” window
- do NOT change the gain during the scanning process since this will change the data resulting in gradient images
- image files will be saved automatically
- after scanning is completed, click on “Plate/Filter” in the “Measurement control” dialog box
- to open the scanner lid click on Plate: “Out”
- remove the slide adapter from the instrument
- to close the scanner lid click on Plate: ”In”
- do NOT forget the slide adpater in the instrument
- close the software and switch of the scanner
- transfer the file to an appropriate folder for data storage. Make sure to arrange for a proper data backup
- do NOT log off or switch off the computer if the HS4800 hybridization station is running
- run image processing software, e.g. GenePix (Axon) or ImaGene (BioDiscovery) for spot identification and signal quantification and perform data evaluation, e.g. using GeneSight (BioDiscovery)

6 Microarray Analysis using the *Medicago* Affymetrix GeneChip

Since September 2005, a 51k Affymetrix *Medicago* GeneChip has been available, following the earlier development of a Symbiosis GeneChip based on a previous TIGR *M. truncatula* Gene Index (Barnett et al. 2004). *Medicago* GeneChips contain probes representing *M. truncatula* EST and genome sequences, and also probes covering the complete genome of *Sinorhizobium meliloti*. The recent Affymetrix *Medicago* GeneChip array available for the general *Medicago* research community was developed from 32,167 expressed sequence tags (cDNAs) in the TIGR *Medicago truncatula* gene index (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago), 18,733 genes predicted from *M. truncatula* genome sequencing that were not represented in the gene index, and 1,896 cDNAs from alfalfa (*M. sativa*) that were not found in the *M. truncatula* sequences. Probe sets in the *Medicago* Affymetrix GeneChip array are composed of 11 pairs of 25-mer oligonucleotides: a perfect match (PM) oligonucleotide and a mismatch (MM) control containing a single nucleotide substitution at the thirteenth base position. The purpose of this

feature is to help distinguish background nonspecific hybridization from true low-level expression of genes (Affymetrix). If one member of the probe set hybridizes aberrantly to the appropriate target, there remains many other probes in that probe set to help determine the expression level for that gene.

Steps in transcriptome analysis using the Affymetrix GeneChip include total RNA extraction, synthesis of double-stranded cDNA, cRNA target amplification and biotin labeling, fragmentation of biotin-labeled cRNA, hybridization, washing, staining and scanning of Genechips. Affymetrix supplies several assay kits and reagents with the purchase of GeneChips. Detailed technical considerations and step-by-step assay procedures can be found in the GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_maual.affx).

We have successfully used 10 µg of total RNA samples from leaf, root and nodule tissues of both *M. truncatula* and *M. sativa* for the production of biotin-labelled cRNA. Fifteen micrograms of fragmented biotin-labeled cRNA is required for the hybridization of the Affymetrix GeneChip *Medicago* Genome Array. The Affymetrix core laboratory facility at the University of Minnesota offers services for the hybridization, washing, staining and scanning steps of GeneChips.

As with other microarray platforms, ensuring that the extracted total RNA is of high quality is an important first step in Affymetrix microarray experiments. The concentration and quality of total RNA as well as biotin-labeled cRNA needs to be determined by several means as RNA degradation leads to poor double-stranded cDNA synthesis and cRNA yield for hybridization. Several options are available for RNA quality and concentration determinations. The first method is UV spectrophotometer absorbance at 260 nm and 280 nm. Acceptable A260/280 nm ratios for good quality total RNA fall in the range of 1.8 – 2.1. Ratios below 1.8 indicate possible protein contamination, while ratios above 2.1 indicate presence of degraded RNA, truncated cRNA transcripts, and/or excess free nucleotides. The quality of extracted RNA can also be assessed by agarose gel electrophoresis in which non-distinct ribosomal RNA bands indicate sample degradation.

Finally, the integrity and quality of extracted total RNA, biotin-labeled cRNA and fragmented cRNA can also be checked with a Bioanalyzer in an RNA Nano LabChip. A good quality total RNA sample should show a low baseline and sharp ribosomal peaks including 18S and 28S.

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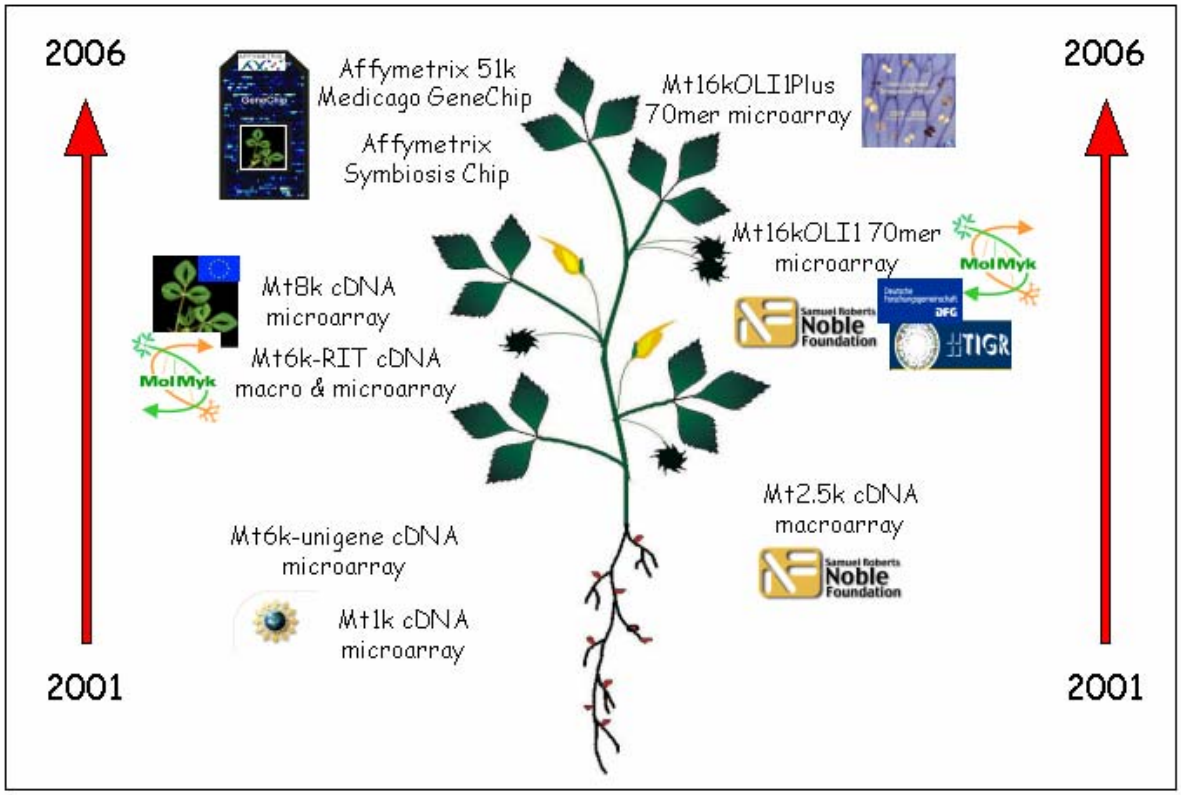

Figure 1: Lineage of *Medicago truncatula* array tools.
Major DNA arrays developed for *Medicago truncatula* are summarized. Contributing institutions, projects, and funding agencies are indicated by the relevant logos. Mt1k, Mt6k-unigene, Lohar et al 2005; Mt2.5k, Liu et al. 2003; Mt6k-RIT, Küster et al. 2004; Mt8k, Firnhaber et al. 2005; Mt16kOLI1 and Mt16kOLI1Plus, Hohnjec et al. 2005; Affymetrix Symbiosis GeneChip, Barnett et al. 2004, Affymetrix *Medicago* GeneChip (http://www.affymetrix.com).