ORDERED DIFFERENTIAL DISPLAY PROTOCOL

(Matz M, Usman N, Shagin D, Bogdanova E, Lukyanov S: **Ordered differential display: a simple method for systematic comparison of gene expression profiles**. *Nucleic Acids Res.* 1997, **25:**2541-2542)

A. Total RNA isolation (for various animal tissues, plants are not tested).

Compiled for tissue volume about 5-15 ul. For larger tissue samples, recalculate the volumes proportionally. No special anti-RNAse precautions (like DEPC treatment of water or tubes) are required. Simply use new batch of tubes and pipetman tips (preferrably plugged), fresh milliQ water for all solutions, and be careful.

Kits involving columns (such as QIAgen RNeasy) are NOT recommended for non-standard objects.

1. On ice, quickly dissolve the tissue in 100 ul of GTC-buffer (4M guanidinium thiocyanate, 25 mM sodium citrate, 30 mM beta-mercaptoethanol, pH 6.5-7.0).
2. Add 100 ul of phenol pH 7.5-8.0 (NOT ACIDIC PHENOL!), mix well (on ice).
3. Add 20 ul of chlorophorm-isoamyl alcohol (24:1), shake vigorously for several seconds 3-4 times, keeping the tube on ice between shakings.
4. centrifugate at 12,000 rpm for 40 min at +4^{o}C.
5. transfer the upper phase (about 80-90 ul) into new tube (be careful not to warm the tube while collecting the upper phase, or else the interphase will disappear), repeat steps 2-4 (if the initial amount of tissue was extremely low, proceed directly to the step 6).
6. Add 1 vol. of ethanol, mix well, immediately centrifugate at 12,000 rpm 10 min.
7. Remove supernatant, wash the pellet once with 200 ul of 80% ethanol (add 80% ethanol, invert the tube gently several times, centrifugate briefly (1 minute at 12,000) and carefully remove supernatant), dry the pellet.
8. *[skip this step if the amount of RNA is expected to be very low – 50-200 ng]* Dissolve the pellet in 10 ul of QT (10 mM tris-HCl pH 8.0; heating at 55^{o}C for 2 min is usually required for complete dissolving), add equal volume (10 ul) of 12M LiCl, mix well and keep at –20^{o}C for one hour. Then centrifugate the sample at maximum speed for 15 minutes (room temp.). Remove supernatant, wash the pellet (it may be invisible) with 200 ul of 80% ethanol once (see step 8), dry it.
9. Dissolve the pellet in 5 ul of QT buffer.
10. Load 1 ul on standard 1.5% agarose gel (for DNA), run until bromophenol migrates about 1.5 cm from well. Take a look to evaluate the RNA amount and

integrity. Ribosomal RNA bands should be well-visible, sharp, with no or very subtle smearing downwards. The band of genomic DNA (nearest to the well) must be much less in intensity than rRNA bands, or invisible. While running the gel, keep the rest of RNA sample on ice.

Each ODD sample requires at least 100 ng of total RNA, though 500 ng is better. If much more RNA is expected from the processed tissue, at the stages 8 and 9 dissolve the pellet in 20 ul of QT, put 5 ul of the final sample into separate tube and treat them as described, to the rest immediately add 1.5 ul 3M NaAcetate pH 5.0 and 45 ul of ethanol, mix well and store at –20^{o}C. To recover RNA from such suspension, simply mix it well, centrifugate the required aliquot at 12,000 for 10 min, remove supernatant, wash once with 80% ethanol, dry and dissolve in QT.

B. Double-stranded cDNA synthesis.

Usually a commercially available kit is used at this step. We use Clontech's Great Length cDNA synthesis kit or Marathon kit. For ODD, one should start the synthesis from the above-mentioned 4 ul of RNA dissolved in QT (containing 0.05-1 mkg of RNA), basically following the company's protocol with the following alterations:

i. The T-primer for first-strand synthesis provided with the kit should be substituted for non-extended ODD T-primer (see oligos list).

ii. for ODD, where the amount of input RNA is low, the T-primer for first-strand synthesis should be put in the reaction in final concentration of 0.3 uM (normally 1 uM concentration is recommended by kit manufacturers)

iii. omit all the stages of "stopping the reaction" if is supposed to be done by adding EDTA or heating above 60C.

Dissolve the cDNA in 20 ul of QT. Do not look at the cDNA at agarose electrophoresis - so far there is nothing informative.

C. cDNA digestion and adapter ligation

In ODD, cDNA species are discriminated by the length of fragment between polyA attachement site and the first occurence of site for some four-base specific restrictase. Two restrictases fit the designed oligos set: *Rsa*I (GT/AC) and *Hae*III (GG/CC). The choice should depend on the average GC-content of cDNA, so that most of cDNA species should produce 3' fragments shorter than 1kb. For most animal species *Rsa*I suits best, though for some special cases with very high GC content *Hae*III may be the enzyme of choice. When more than 300 ng of RNA was put into cDNA synthesis, the volume of cDNA solution may be adjusted to 40 ul by QT and the following two steps

(digestion-ligation and PCR) may be made in parallel with two different restrictases, to see which would produce the amplified cDNA preparate consisting of shorter fragments.

1. To cDNA dissolved in 20 ul QT add 2.2 ul of reaction buffer provided with the restrictase and 1.5 ul of the restrictase. Incubate at 37^{o}C for 1.5 hours.
2. Inactivate the restrictase by heating (for *Rsa*I, 2 min at 60^{o}C) and phenol-chlorophorm extraction, precipitate the cDNA by ethanol-NaAc, wash once with 200 ul of 80% ethanol, dry and dissolve in 5 ul QT.
3. Add 2 ul of 10 uM ODD adapter (mixture of two adapter oligos 10 uM each, see oligos list), 1 ul of 10x ligation buffer (provided with the ligase) and 1 ul (1-5 u) of ligase. Leave the reaction at 16^{o}C overnight.
4. Add 90 ul of water to the ligation mixture and purify it by QiaQuick-spin PCR purification column, according to the provided protocol for PCR products. Elute with 30 ul of T&M solution (10 mM tris-HCl pH 8.0, 1 mM $MgCl_2$) - NOT WATER! This stage is required to remove the residual adapter oligos, so that larger volume of ligated cDNA could be put into the subsequent PCR. It is essential when the initial amount of RNA was very low (100-200 ng). Alternatively, the ligation mixture may be diluted five-fold with water and 1 ul of this dilution may be taken for subsequent PCR.

D. Amplification of basic samples.

This is the key step in the whole procedure. The product of amplification (smear with some bands) should become well-visible on agarose gel (i.e. reach the DNA concentration of about 25 ng/ul) after not more than 15-18 PCR cycles, to be sure that the cDNA samples are well-representative. Poor representativity of the samples obtained in more than 20 PCR cycles leads to the great increase of "statisitical noise" during display, which may lead to false positives. All the samples processed in parallel should have the same look on agarose gel, showing the same smear-band pattern. Slight difference in the product abundances (2-4 fold) does not affect the analysis and can be equalized by 1-2 additional PCR cycles performed for less abundant samples or by the extent of subsequent dilution.

It is very important to use KlenTaq (Ab Peptides Inc.) or its analog in all the ODD PCRs. No other polymerase makes it possible to obtain such clear and reproducible pictures as KlenTaq.

PCR mixture (for one sample) contains:

10x PC2 buffer (200 мМ tricine-KOH pH 9.1, 160 мМ ammonium sulphate, 30 мМ $MgCl_2$, 0.5 мg/ml BSA)	2.5 ul
5 mM dNTPs	0.5 ul
10 uM non-extended T-primer	0.25 ul
10 uM DAd-primer	0.25 ul
Deionized water	12 ul
KlenTaq 25 u/ul (Ab Peptides)	0.25 ul
QIA-purified cDNA sample	10 ul

So, the total volume of the reaction is 25 ul, and 10 ul of these are the QIA-purified ligated cDNA sample. If no QIA purification was made, 1 ul of five-fold dilution of the ligated cDNA sample is added to 24 ul of the mixture (adjust the volume with deionized water).

The above mixture is the same for all the samples for ODD comparison. It is perpared in large volume and then subdivided into aliquots, to which ligated cDNA is added separately.

Cycling conditions:

temperature control in the reaction tube or simulated tube control (Hybaid, MJ research cyclers):

95^oC 5 sec – 65^oC 30 sec – 72^oC 1 min

block control (old Perkin-Elmer cyclers):

94^oC 40 sec – 65^oC 30 sec – 72^oC 1.5 min

At first, 15 cycles should be performed. After this, 3 ul of each reaction are loaded on agarose electrophoresis and run for a short while - about 1 cm from wells (meanwhile the reactions may be kept on table). If some or all products are poorly visible, put the corresponding samples back into thermocycler and perform additional 1-3 cycles. To estimate the required number of additional cycles, it can be roughly supposed that each PCR cycle raises the product amount twofold. If a product is just barely visible even on such a short run, the sample should be given 4 more cycles. Then the products are loaded on gel again and their relative amounts evaluated.

The smears should be mostly below 1 kb, maximum of their intensity shoud appear at 300-700 bp range. If the smears are high and the maximum is at about 1 kb or higher, alternative resriction enzyme (stage C) should be considered.

The obtained PCR products should be diluted 30-100-fold in QT with 20 ng/ul purified yeast tRNA added. The exact extent of dilution is selected in such a way that all the diluted samles would contain DNA in the same concentration. The diluted samples serve as a basic cDNA source for ODD patterns production. They can be stored at –20^{o}C for years.

E. ODD reactions

ODD PCR products may be labelled by using in-strand incorporation as well as by terminal labelling of PCR primer. We recommend the latter, because of two reasons: first, in this way only one strand of the PCR product become labelled and visible on gel, which to some extent lowers the complexity of the pattern; and second, PCR products appear on gel with the intensity depending solely upon the cDNA species' concentration in the sample. With in-strand labelling, the intensity depends also upon the length of the fragment, so that the shorter products become much less pronounced than the longer ones. As for the isotope, ^{33}P is highly recommended.

Terminal labelling:

Only one primer is labelled, the one which participates in all primer combinations being tested.

Extended primer phosphorilation, for ten ODD reactions:

10 uM extended primer	2 ul
10x polynucleotide kinase buffer (500 mM tris-HCL pH 7.6, 100 mM $MgCl_2$, 10 mM spermidine, 10 mM EDTA)	1 ul
γ^{33}P-ATP 10 uCi/ul (3000-5000 Ci/mmole)	5 ul
Water	1 ul
T4 polynucleotide kinase (5 u/ul)	1 ul

Incubate 30 min. at 37^{o}C, then stop the reaction by heating for 2 min at 70^{o}C.

ODD subset amplification:
Reaction mixture for 10 reactions:

10x PC2 buffer (see section D)	10 ul
5 mM dNTPs	4 ul
Kinase reaction	10 ul
10 uM second (not labelled) extended primer	2 ul
Water	74 ul
TaqStart antibodies (Clontech)	3 ug
KlenTaq 25 u/ul (Ab Peptides)	1 ul

The mixture is dispensed into 9 ul aliquots and 1 ul of diluted basic samples (stage D) is added to each. PCR is then performed according to the same cycling conditions as described at stage D, for 25 cycles.

In-strand labelling:

Reaction mixture for 10 reactions:

10x PC2 buffer (see section D)	10 ul
5 mM dNTPs	2 ul
10 uM TE-primer	2 ul
10 uM InE-primer	2 ul
Water	79 ul
α^{33}P-dATP 10 uCi/ul (3000-5000 Ci/mmole)	5 ul
TaqStart antibodies (Clontech)	3 ug
KlenTaq 25 u/ul (Ab Peptides)	1 ul

The mixture is dispensed into 9 ul aliquots and 1 ul of diluted basic samples (stage D) is added to them. PCR is then performed according to the same cycling conditions as described at stage D, for 25 cycles.

It is recommended, before preparing labelled reactions, to test several extended primers combinations without labelling (PCR as just described but without radioactivity) and look at the products on agarose gel. The products should be well-visible after 23-25 PCR cycles, have the same intensity within a particular primers combination, and substantial pattern differences should be observed between primer combinations.

E. Electrophoresis

Ordinary 0.4mm-thick 5% sequencing gel is used. The buffer is 1x TBE in gel and lower chamber, 0.5x TBE in upper chamber (1x TBE: 0.089 M tris-borate, 2 mM EDTA, pH 8.5)

5 ul of labelled ODD reaction are mixed with 5 ul of standard sequencing stop-solution. These samples are heated at 90^{o}C for 5 minutes before loading (do not let the samples evaporate while heating). 3-5 ul are loaded on each lane.

Before loading, special pre-run is required: upon removing the comb from the gel, wash wells with 0.25x TBE (twice-diluted upper chamber buffer), load reference (2 ul of stop-solution) into one well and run the gel at maximum voltage until bromophenol migrates about 0.5 cm. Then wash wells again with 0.25x TBE and load the samples. Run-in (until both dyes migrate into the gel) at 1/3 of maximum voltage, then switch to maximum. Run the gel until xylene-cyanol migrates about 2/3 of the gel length.

The pre-run is needed to create a buffer gradient under the wells, so the samples become concentrated as they migrate into gel. This greatly improves the resolution.

After running, dry the gel on whatman paper at 60^{o}C and expose to X-ray film overnight. Attach fluorescent or radioactive position markers to the gel so the autograph could be aligned with the gel for band excision.

F. Band excision and amplification.

1. Align the autograph with the gel. Using hot needle, pierce the film twice to mark the middle 3/4 of the band of interest (do not take the edges of the band!). Remove film, excise the piece of the gel-paper sandwich surface between the two marks. Cut as thin as possible. **As a negative control, cut the same region of the neighboring lane which does not contain the band** (this is especially important for weak differential bands).
2. Put the excised piece into 30 ul of QT, incubate the tube at 55^{o}C for 2 hours or at +4^{o}C overnight.
3. Take 1 ul of the eluate for PCR with corresponding extended primers (in 25 ul). PCR conditions are the same as described for basic samples' production (section D). Intense and short (meaning the fragment length) bands are usually amplified in 15-17 cycles, long and weak ones - in 19-22 cycles. Do not make too much cycles - start from 15, then look at the aliquot (3 ul) on agarose gel. If there is nothing - make 5 more cycles, if something barely visible - 3 more cycles.

If the product of re-amplification is well-seen at agarose after 20 cycles or less and looks like a single band, while the negative control is empty, such product is likely to be successfully sequenced directly by any method suitable for PCR products using extended primers. However, not to take any chances with direct sequencing, we recommend to clone the product, and take the clones containing the most frequent insert (which is the target one) which may be identified in the following way. After cloning, colonies are usually tested by means of PCR with vector-specific primers to select clones containing inserts of correct length. After selecting several (5-7) inserts of correct length, 2 ul of the same PCR product is put into 10 ul of restriction mixture, containing coctail of four-cutters: *Mbo*I, *Alu*I and *Hae*III (or *Rsa*I, if *Hae*III was used in ODD), in NEBuffer 2 (New England Biolabs). The products of restriction (all 10 ul) are resolved on (3% NuSieve GTG/1% standard agarose) or 2.5% Metaphor agarose. The identical clones can be identified as those producing identical band patterns.

A helpful improvement to the band cloning procedure was suggested by Yoshikatzu Hirate (Lab. for Developmental Gene Regulation, RIKEN Brain Science Instutute; hirate@brain.riken.go.jp). To separate the target band from the background redundant fragments of the same size before cloning, the products of re-amplification from target band and negative control (see above, F-1) are run side-by-side on agarose in the presence of H-A Yellow reagent. H.A.-Yellow is a product of Hanse Analytik (in japan, it is dealt with by TAKARA Shuzo), which selectively binds to AT base pairs. By adding the reagent in agarose gel, loaded DNA fragments are separated according to their AT contents. Thus, the target band will separate from background ones, even if it has the same length. The presence of a negative control on the neighboring lane allows to discriminate the target band in the pattern and pick it out from agarose gel.

G. Checking the differential sequences.

Primary prove of band "differentiality" is provided by the reproducible pattern seen in parallel duplicates already on ODD gel. Only "reproducibly differential" bands should be excised and analysed.

In our opinion, the easiest way to check the sequence for being differential is RT-PCR with the primers synthetized for the sequence. It is also the most sensitive method. Alternatively, Northern blot hybridization may be used. It is respectable, but requires tremendous amounts of RNA and still much less sensitive than RT-PCR. It should be noted that the product of band re-amplification cannot be used to prepare a probe for hybridization (too much background), cloned sequence is required.

First of all, PCR with band-specific primers should be performed on the basic samples used for comparison. Careful cycling is required, because over-amplification may equalize the original difference between the samples. Most bands are amplified in 20-27 cycles starting from 1 ul of diluted basic ODD sample, so use the same “careful cycling” strategy to avoid over-amplification as when re-amplifying excised bands, but start looking at the products at 20 cycles.

After getting positive result with ODD samples, RT-PCR should be performed on independently isolated RNA samples not used in ODD.

Please forward your remarks and questions to Mikhail Matz (matz@whitney.ufl.edu)

ODD OLIGOS LIST

Adapter (10 uM mixture of two oligos):
5'- tgt agc gtg aag acg aca gaa agg gcg tgg tgc gga ggg cggt
5'- acc gcc ctc cg

Distal adapter-specific primer (DAd):
5'- tgt agc gtg aag acg aca gaa

Non-extended T-primer:
5'- cgc agt cga ccg (t)13

Inner adapter-specific extended primers (InE-primers)
(for HaeIII and/or RsaI digested cDNA)
Bases which are specially importaint are typed IN BIG LETTERS.

5'- gcg tgg tgc gga ggg cggt gc GG
5'- gcg tgg tgc gga ggg cggt gc GA
5'- gcg tgg tgc gga ggg cggt gc GT
5'- gcg tgg tgc gga ggg cggt Tc GC<--NB! G->T substitution!

5'- gcg tgg tgc gga ggg cggt gc AG
5'- gcg tgg tgc gga ggg cggt gc AA
5'- gcg tgg tgc gga ggg cggt gc AT
5'- gcg tgg tgc gga ggg cggt Tc AC<--NB! G->T substitution!

5'- gcg tgg tgc gga ggg cggt gc TG
5'- gcg tgg tgc gga ggg cggt gc TA
5'- gcg tgg tgc gga ggg cggt gc TT
5'- gcg tgg tgc gga ggg cggt gc TC

5'- gcg tgg tgc gga ggg cggt gc CG
5'- gcg tgg tgc gga ggg cggt gc CA
5'- gcg tgg tgc gga ggg cggt gc CT
5'- gcg tgg tgc gga ggg cggt Tc CC<--NB! G->T substitution!

---------- || ^^---extention for ODD
||
two bases covering
the restriction site
(with mismatch, suitable
for Rsa I and Hae III cleaved DNA)

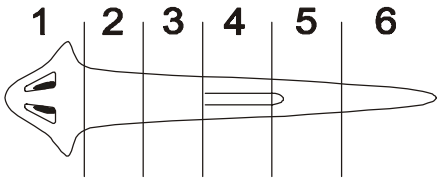
Extended T-primers (TE-primers):

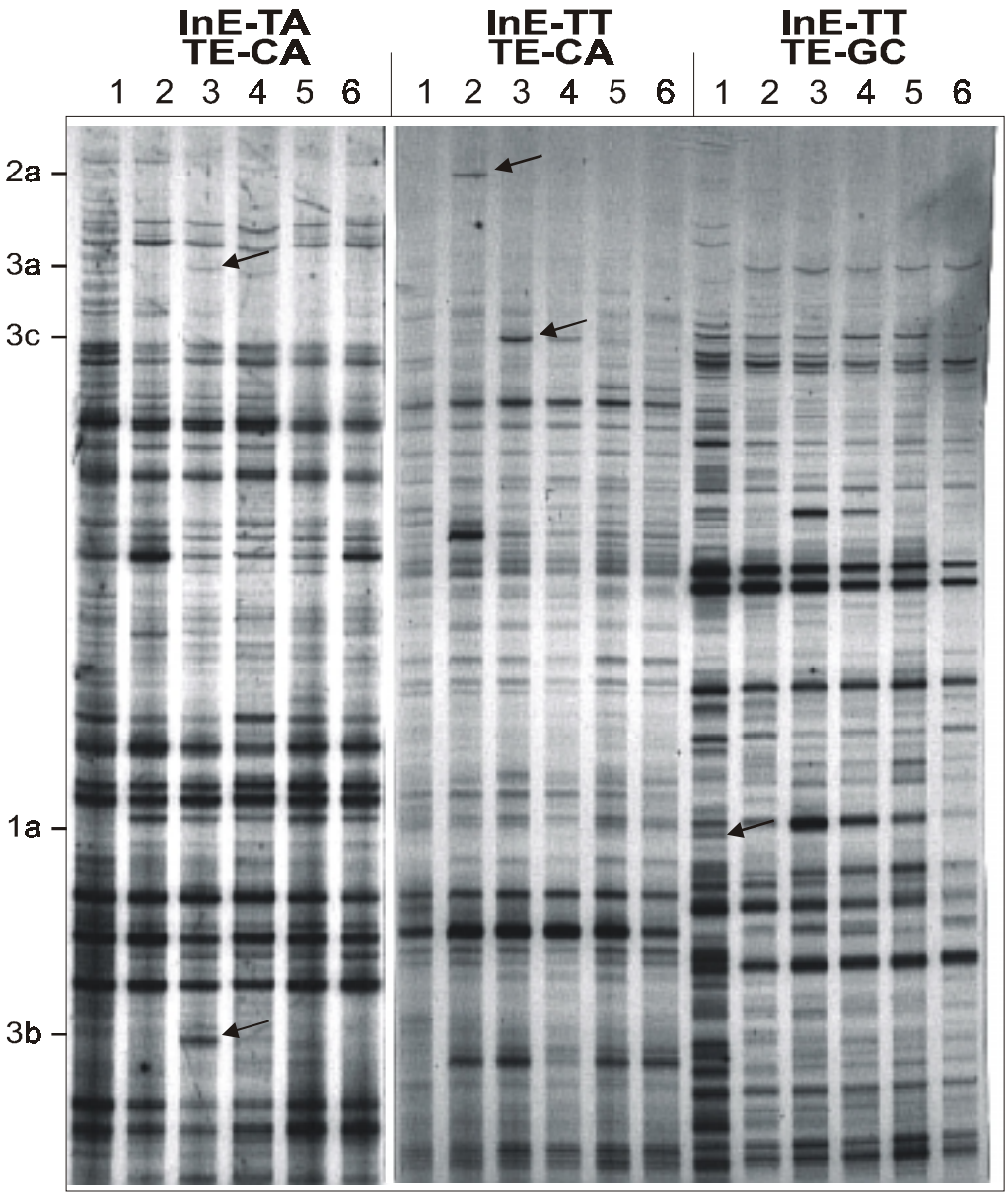
5'-cgc agt cga ccg (t)13 AG
... AA
... AT
... AC

And so on, all extention variants except those with T in the first position.

ALL OLIGOS MUST BE HPLC-PURIFIED !!!

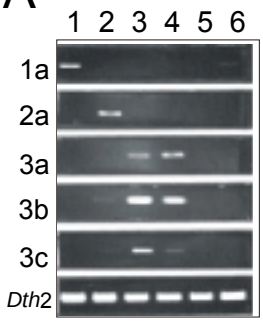
Regional markers of planarian





Regional markers of planarian
confirmation of differential distribution

A



A - RT-PCR.

B - *in situ* hybridization.

B