Representational Difference Analysis Protocol.

Foreword

It is very important that the two different populations of cDNAs (which are prepared from RNA isolated from two different sources) be kept separate and distinct from each other throughout the analysis. Any cross-contamination of the samples will skew the RDA by introducing artifacts which will likely lead to the cloning of false positives. It is strongly recommended that a color-coded microfuge tube system be adopted to ensure that the samples are not mixed up and inadvertently cross-contaminated during the numerous pipetting operations required for the RDA procedure.

Further, to reduce the likelihood of outside contamination, aerosol-resistant pipette tips must be used at all times. Extreme care must be taken when pipetting core mixes into multiple tubes, since a contaminated tip can ruin the whole RDA. Gloves, changed often, will also help to keep contamination from occurring. Finally, it is crucial that all reagents, plastic ware, etc. be RNA/DNA/Nuclease free. Careful attention to these and other details will go a long way to help ensure a successful RDA.

A. Isolation of total RNA / DNase treatment

It is crucial that the RNA used for RDA be undegraded and free of DNA or other contaminants. We have been very successful using Trizol (Gibco / Life Technologies) for the isolation of total RNA. Trizol, a proprietary mix of guanidinium isothiocyanate and phenol, allows for the quick and economical isolation of total RNA from either tissue samples or cells grown in culture. Further, it is important that enough RNA is isolated in order to perform the RDA as well as any Northern analyses that are needed to verify the differential expression(s) of the cDNA fragments cloned by RDA. If possible, we recommend that 500ug of total RNA be isolated in order to meet these criteria. Generally, this amount of RNA can be isolated from eight to ten 100mm cell culture dishes. In the following protocol the approximate yields expected at key steps of the RDA are detailed based on 250ug of total RNA for each treatment.

It is advisable to treat the total RNA with RNase-free DNase to ensure that all residual DNA is removed before proceeding with the RDA. For DNAse treatment of total RNA, a typical reaction consists of the following:

250ug	Total RNA (diluted to 1 ug/ul)
50ul	10X DNase I buffer
2.5ul	Dnase I (10 units/ul) (Epicentre)
197.5ul	Dnase/Rnase Free Water
500ul	Total for each RNA population

Incubate reactions at 37°C for 1hr

Add 50ul 10X Termination Mix (0.1M EDTA [pH 8.0]; 1mg/ml Glycogen)

Extract with 550ul Phenol:Choloroform:Isoamyl alchohol (25:24:1) (referred to hereafter as PCI)

Remove upper aqueous layer and precipitate RNA using 100ul 10M NH_4OAc and 750ul absolute ethanol

B. Preparation of Poly (A) RNA

Prior to cDNA synthesis, the poly(A) RNA must be separated from the rRNAs and tRNAs. In our laboratory, we use oligo dT latex beads to capture the poly(A) RNA (Qiagen Oligotex Kit). Following the protocol provided with the kit, we have found that 250ug of total RNA will yield approximately 5-10ug of pure poly(A) messenger RNA. The freshly isolated poly(A) RNA is eluted in a small volume (30ul) so that the entire solution may be used directly in subsequent cDNA synthesis. Because poly(A) RNA is labile and subject to degradation while in solution, it is very important that cDNA synthesis is begun immediately following the poly(A) RNA isolation step (do not store the Poly(A) RNA).

C. & D. Reverse Transcription of mRNA into cDNA

A number of suitable cDNA synthesis kits are available to convert the poly(A) RNA to double stranded cDNA. The Riboclone System (Promega) is used for cDNA synthesis in our laboratory, and by following the manufacturers protocol, results in 50%-60% total conversion efficiency from poly(A) RNA to double stranded cDNA. Here we present only highlights of the required reactions for complete cDNA synthesis as numerous detailed protocols already exist. Typically, first strand synthesis is allowed to proceed for 90min–2hr to maximize the length of the nucleic acid strands and during the last 15min of the reaction the temperature slowly increased from 42°C to 50°C to increase the efficiency of reverse transcription of regions containing strong secondary structure. Second strand cDNA synthesis immediately follows the reverse transcription of the RNA using E. coli DNA polymerase I and proceeds for up to 6hr, completing the conversion of mRNA templates to full-length cDNAs (1–5ug of cDNA from each population is desirable for RDA analysis) .

Following cDNA synthesis, aliquots of each full-length double-stranded cDNA reactions (approximately 1.5µg) is analyzed on a 1% agarose gel to confirm the integrity of the cDNA populations to be compared by RDA. Ethidium Bromide staining should reveal a smear beginning at about 300bp and extending up to 7000bp, with the greatest intensities at 2000-3000bp. It is most important that the smears from both samples appear identical based on the size distribution.

E. Digestion of cDNA with DPN II

Amplicon preparation begins with digestion of approximately half of each cDNA population with the restriction enzyme DPN II (the other half of the cDNA population is saved for future library preparation and screening).

5ul	cDNA (1-5 ug)
10ul	10X DPN II buffer
3ul	DPN II (10 units/ul) (New England Biolabs)
82ul	Dnase/Rnase Free Water

100ul Total for each digestion reaction

Incubate reactions at 37°C for 1hr

Extract with 100ul PCI

Remove upper aqueous layer (Discard organic layer)

Extract 100ul chloroform

Remove upper aqueous layer containing digested cDNA

Following chloroform extraction, each of the DPNII-digested cDNA populations is immediately passed through separate spin columns containing either sephacryl S-300 (Amersham/Pharmacia) or using commercially prepared PCR Select-II columns (5 Prime-3 Prime).

Spin at 500xg for 5min (gel filtration spin columns efficiently remove all cDNA species smaller than 100bp)

Precipitate cDNA eluates with 100µl of 10M Ammonium Acetate and 750 µl of absolute ethanol

Spin all tubes at high speed (12,000xg) for 15min at 4°C to pellet the digested cDNA

Decant the supernatants and gently wash the pellets with 100µl ice-cold 70% ethanol

Driver Synthesis

F. Ligation of Driver PCR Primers

Remove all of the ethanol from the digested cDNA pellets and resuspend each sample in 20µl of TE buffer

Prepare a core mix of primers and ligation buffer by combining in a separate tube:

10X Ligation Buffer	9µl (supplied with ligase enzyme)
Driver 24mer Primer	9µg
Driver 12mer Primer	4.5µg

Add water to bring the total volume to 45µl

Add 20µl of the core mix to each of the tubes containing the resuspended (DPN II-digested) cDNAs

Place the tubes in a preheated 55°C heat block. Remove the heat block containing the samples to the cold room and allow it to cool slowly to <10°C to allow the 12mers and 24mers to anneal and form cohesive ends complementary to the DPN II sites on the ends of the digested cDNAs (45min–1hr)

Spin the samples briefly to collect all of the condensation and return them to the cooled heat block

Make another core mix containing:

10X Ligation Buffer	11µl	
Water	90µl	
T4 DNA Ligase	9µl	(New England Biolabs 400 Cohesive end units/µl)
Total volume to 100µl		

Add 50µl of the core mix to each of the cooled tubes containing the cDNA and primers and mix well

Incubate the tubes overnight at 15°C in order to ligate the 24mers onto the 5'ends of the cDNAs

The next morning, incubate the tubes at 65°C for 10min (inactivate ligase)

After heating each ligated cDNA population is passed through separate spin columns as described previously

Spin at 500xg for 5min (gel filtration spin columns efficiently remove all cDNA primers and ligase)

Transfer the eluted, 24mer primer-ligated cDNAs to labeled 1.7 ml microfuge tubes

Adjust the volumes of the eluates to 100µl (Keep on ice)

G. PCR Amplification of Driver

Label and set aside six PCR tubes for each cDNA population (e.g. 6 Red and 6 Green tubes)

In a sterile disposable culture tube, make a core mix by adding:

10X PCR Buffer	250µl
25mM $MgCl_2$	400µl
5mM dNTPs	125µl
Water	1425µl
Total Volume	2200µl

Mix well and remove 240µl to a new microfuge tube labeled "Taq" and place on ice. (This precaution ensures that the Taq enzyme and the primers are not combined at temperatures below 72°C and eliminates the possibility of primer:dimer formation and the undesired extension of these dimers by Taq)

To the remaining 1960µl in the culture tube, add 25µg of Driver PCR primer (the same 24mer that was used in the ligation step, but without the 12mer) and enough water to bring the total volume to 2060µl.

Transfer half the contents of the culture tube (1030µl) into each of the tubes containing the eluted, ligated cDNAs. Mix each tube well and centrifuge briefly.

Into each of the appropriate pre-labeled PCR tubes, transfer 180µl of the core mixes containing the cDNAs. (Disregard the few microliters that will be left over)

Add 13µl of Taq enzyme (5 units/µl) to the "Taq" tube that has been sitting on ice.

Transfer the tubes to a thermal cycler pre-heated to 72°C and incubate 1-2 min. (Fully extends the cDNA and creates PCR primer binding sites) (Do not heat to 95°C, which would denature the DNA and render it incapable of being PCR amplified)

Add 20µl of the "Taq" mixture to each of the PCR tubes and mix by pipetting several times. (Do not let the tubes cool below 72°C)

Overlay the samples with 50µl of mineral oil (if necessary) and incubate the PCR tubes that now contain Taq for an additional 5min at 72°C.

Following the 5min incubation with Taq, immediately begin PCR cycling:
95°C 45 sec
72°C 4 min
for 25 Cycles

After the cycles are completed, incubate the tubes for 10min at 72°C (Ensures all of the species are double-stranded and fully extended)

Following PCR, pool the six 200µl samples from cDNA population #1 in one microfuge tube and pool the six samples from cDNA population #2 in a separate microfuge tube.

Extract each pooled sample with 700ul PCI

Remove upper aqueous layer and divide them evenly among four new microfuge tubes. (approximately 300µl into each tube for each cDNA population)

Precipitate cDNA with 150µl of 10M Ammonium Acetate and 1200µl of absolute ethanol. Let tubes cool at -20°C for at least 1hr

Spin all tubes at high speed (12,000xg) for 15min at 4°C to pellet the cDNA

Decant the supernatants and gently wash the pellets with 100µl ice-cold 70% ethanol

Remove the supernatant completely and allow the pellets to air dry for 1-2min

Resuspend each of the pellets in 25µl of TE buffer. Pool the like samples to yield one tube for each cDNA population containing 100µl.

H. Removal of Driver PCR Primers by DPNII

Transfer 90µl of each of the driver DNA into clean tubes.
Into each tube, add:

10X DPN II Buffer	10.5µl
DPNII Enzyme	4.5µl
Total Volume	105µl

Incubate the tubes at 37°C for 3hr to completely restrict the driver PCR primers.

Extract each sample with 100ul PCI

Remove upper aqueous layer

Extract 100ul chloroform

Remove upper aqueous layer and pass through separate spin columns (as described above)

Precipitate cDNA eluates with 100µl of 10M Ammonium Acetate and 750µl of absolute ethanol

Spin all tubes at high speed (12,000xg) for 15min at 4°C

Decant the supernatants and gently wash the pellets with 100µl ice-cold 70% ethanol

Remove residual ethanol from each sample and resuspend the driver pellets in 75µl of TE buffer

Quantitate the amounts of driver DNA by measuring absorbance at 260nm. (Typically we dilute 5µl of each of sample into 395µl of TE buffer in a 1:80 dilution giving absorbances between 0.1 and 0.2. An absorbance of 1.0 at 260nm correlates to DNA concentrations of 50µg/ml)

After calculating the concentrations of each of the driver cDNA stock solutions, adjust the volume of the more concentrated sample (by adding TE buffer) to make the concentrations of both tubes equal.

Run 1µg of each driver on a 1% agarose gel stained with ethidium bromide. Both samples should appear to be identical smears ranging in size from about 150bp to 600bp, with some faint smearing extending up to about 1000bp. Yield should be 50-75ug per tube of cDNA.

I. & J. Ligation of RDA PCR Primers. (Tester cDNA Preparation)

This ligation is performed in the same manner as was the ligation that is described above in section F.

Label two new tubes and transfer 1µg of the appropriate driver cDNA. Adjust the volume of each tube to 20µl by adding the proper amount of TE buffer.

Make a core mix of primers and ligation buffer by combining in a separate tube:

10X Ligation Buffer	9µl
Round One 24mer Primer	9µg
Round One 12mer Primer	4.5µg
Total Volume	45µl

Add 20µl of the core mix to each of the tubes containing the resuspended driver cDNAs.

Place the tubes in a preheated 55°C heat block. Remove the heat block containing the samples to the cold room and allow it to cool slowly to <10°C (45 min–1 hr).

Spin the samples briefly and return them to the cooled heat block.

Make another core mix containing:

10X Ligation Buffer	4.5µl
Water	34.5µl
T4 DNA Ligase (New England Biolabs)	6µl
Total Volume	45µl

Add 20µl of core mix to each of the cooled tubes containing the cDNA and primers

Incubate the tubes overnight at 15°C

(These ligated cDNAs will become the tester population for the first round of RDA subtraction).

K. L. M. N. Subtractive Hybridization of Species Common to both cDNA Populations.

We have found in our own experiments that an initial ratio of 1:10 followed by ratios of 1:100, 1:5,000, and 1:25,000 will almost always result in a successful RDA yielding differentially expressed cDNAs (given that the driver populations do in fact contain differentially expressed species.) The hybridization protocol that follows thus reflects this series of ratios.

Label two new tubes using a color coordination scheme help insure that the two cDNA populations are appropriately identified throughout the rest of the procedure (Red-tester cDNA population #1 (e.g. treated) / Green-tester cDNA population #2 (e.g. untreated).

Heat the ligation mixes to 65°C for 10min (comprising tester cDNA from previous step).

Transfer 30µl (0.5µg) of the ligation mix from cDNA population #1 to the Red tube. Similarly, transfer 30µl (0.5µg) of the ligation mix from cDNA population #2 to the Green tube.

Into the Red tube, transfer 5µg of Green driver DNA. Similarly, into the Green tube, transfer 5µg of Red driver. Adjust volumes in each tube to 50µl with TE buffer.
Note that this is the only time when opposite cDNAs are mixed (Figure 2/Step K).

Extract each sample with 50ul PCI

Remove upper aqueous layer (discard organic layer)

Extract 50ul chloroform

Remove upper (aqueous) layer transfer to fresh microfuge tube.

To each of the tubes containing the tester:driver mixes, add:
10M Ammonium Acetate 50µl
Absolute Ethanol 375µl

Place the tubes in the freezer at –20°C for 2hr to co-precipitate the tester:driver cDNAs

Spin all tubes at high speed (12,000xg) for 15min at 4°C

Decant the supernatants and gently wash the pellets with 100µl ice-cold 70% ethanol

Remove residual ethanol from each sample air dry cDNA pellets for 1-2min

Resuspend the cDNA pellets in 4µl of hybridization buffer (Insure cDNAs are completely dissolved in the buffer by repeatedly pipetting up and down and by vortexing the samples)

Centrifuge the tubes briefly to collect the contents at the bottom

Into two new tubes, (one Red and one Green) transfer 1µl of 5M NaCl

Place the tubes into a thermal cycler that has been pre-heated to 95°C. Incubate the tubes containing the tester:driver cDNAs for 1min in a pre-heated (95°C) thermal cycler to denature the cDNAs.

Centrifuge tubes briefly after heating to collect the contents

Immediately transfer the denatured cDNAs to the appropriate new tubes containing the NaCl and mix by repipetting several times

Add 20µl of mineral oil to each of the tubes

Centrifuge briefly to collect the contents

Incubate the tubes containing tester:driver cDNAs with NaCl in the thermal cycler for an additional 3min at 95°C to ensure that all cDNA species are completely denatured

Set the thermal cycler to soak at 67°C (with the samples still in the machine)

Incubate the samples at 67°C for at least 18hr (and not more than 48hr) to allow the cDNAs to hybridize with their complementary strands

O. PCR Amplification of Subtracted Tester cDNA.

Following hybridization of the tester:driver cDNAs, carefully remove as much of the mineral oil as possible. (without removing any of the hybridization buffer)

Add 45µl of water to each of the tubes containing the hybridized DNAs

Label two new PCR tubes (one Red and one Green) with the number "1" on each tube. Transfer 10µl (1/5 of the total contents) of the hybridized tester:driver DNAs to the appropriate new tubes (e.g. Red to Red and Green to Green).

Label two new 1.7ml tubes, one of them "mix" and the other "Taq".

Into the "mix" tube, make a core mix by adding:

10X PCR Buffer	44µl
25mM $MgCl_2$	70µl
5mM dNTPs	22µl
Water	232µl
Total Volume	368µl

Transfer 44µl of this mix to the tube labeled "Taq"

To the "mix" tube, add 4.5µg of round one PCR 24mer primer in a vol of 50µl to bring the total volume in the mix tube to 374µl

Transfer 170µl from the "mix" tube into each of the PCR tubes containing the 10µl of tester:driver DNAs

Incubate the PCR tubes at 72°C (**Do not heat to 95°C)**

To the "Taq" tube, add 2.5µl of Taq polymerase (5units/µl)

Transfer 20µl from the "Taq" tube to each of the tubes that are incubating at 72°C

Return the tubes to the 72°C (Do not allow the samples to cool)

Overlay the samples with 50µl of mineral oil (if necessary) and incubate the samples for an additional 5min following addition of the "Taq" mixture to extend the cDNAs and create PCR primer binding sites.

After the 5min incubation, immediately begin PCR cycling
95°C 45 sec
72°C 4 min
For 7 cycles

Label and set aside 8 new PCR tubes (4 Red and 4 Green) with the number "2".

As before, label two new 1.7 ml tubes with "mix" and "Taq"

In the "mix" tube make a new core mix by adding:

10X PCR Buffer	170µl
25mM $MgCl_2$	272µl
5mM dNTPs	85µl
Water	1123µl
Total Volume	1650µl

Transfer 180µl of this mix to the "Taq" tube

To the "mix" tube, add 17µg of round one PCR 24mer primer in a volume of 50µl to bring the total volume in the mix tube to 1520µl

Transfer 180µl from the "mix" tube into each of the eight new PCR tubes (4 Red and 4 Green)

During the seventh PCR round, transfer 8.5µl of Taq polymerase (5U/µl) to the "Taq" tube.

During the 72°C extension step of the seventh round of PCR, pause the thermal cycler at 72°C and insert the four Red and four Green tubes (labeled "2") into the thermal cycler. (Leave the first tubes in the thermal cycler as well)

Add 20µl of the "Taq" mix tube to each of the eight #2 tubes. (Temperature should remain at 72°C during this step and the next)

Transfer 10µl from each of the #1 tubes to the corresponding #2 tube (i.e. 10µl from the Red #1 tube into each of the four Red #2 tubes, for a total of 40µl transferred. Do the same for the Green tubes.) Overlay the samples with 50µl of mineral oil (if necessary) and proceed immediately to PCR.
95°C 45 sec
72°C 4 min

For 20 cycles.

Incubate the tubes for 10 minutes at 72°C

Following PCR, pool the same-color samples from the tubes labeled #2 (disregard the #1 tubes) into 1.7ml microfuge tubes of corresponding colors

Extract each pooled sample with 700 µl of PCI

Carefully remove the top (aqueous) layers and divide them evenly among six (three Red and three Green) new 1.7ml tubes (approx 260µl in each tube)

To each of the six tubes, add:

10M Ammonium Acetate	140µl
absolute ethanol	1200µl

Precipitate cDNA at –20°C for at least 2hr

Spin all tubes at high speed (12,000xg) for 15min at 4°C.

Decant the supernatants and gently wash the pellets with 100µl ice-cold 70% ethanol

Remove residual ethanol from each sample air dry cDNA pellets for 1-2min

Resuspend each of the pellets in 33µl of TE buffer

Pool like samples to yield one tube for each treatment

P. Removal of PCR Primers by DPNII

Transfer 87µl of each of the PCR'd tester DNAs into clean, appropriately colored tubes

To each tube, add:

10X DPN II Buffer	10µl
DPNII Enzyme	3µl
Total Volume	100µl

Incubate the tubes at 37°C for 3 hr

Extract each sample with 100ul PCI

Remove upper aqueous layer (discard organic layer)

Extract 100ul chloroform

Remove upper aqueous layer and pass through separate spin columns (as described above)

Precipitate cDNA eluates with 100µl of 10M Ammonium Acetate and 750µl of absolute ethanol

Spin all tubes at high speed (12,000xg) for 15min at 4°C

Decant the supernatants and gently wash the pellets with 100µl ice-cold 70% ethanol

Remove residual ethanol from each sample and resuspend the driver pellets in 75µl of TE buffer

Quantitate the amounts of driver DNA by measuring absorbance at 260nm

After calculating the concentrations of each of the driver cDNA stock solutions, adjust the volume of the more concentrated sample (by adding TE buffer) to make the concentrations of both tubes equal. Yield should be 10-20ug per tube of digested Tester cDNA.

Analyze 1µg of each product on a 1% agarose gel stained with Ethidium Bromide. Some distinct bands may be visible after round one, but more likely, no bands will be observable until after the third round of RDA.

Q. RDA Rounds Two Through Four.
In each successive round of RDA, the product of the previous round serves as the tester for the next round. For example, the digested product (enriched for differentially expressed species) of round one will serve as the tester for round two; the product of round two will be the tester for round three, and so on. In each round of RDA, the amount of driver cDNA used will remain constant (5µg for this protocol), while the amount of tester will diminish in each round. The amount of tester used is subject to the hybridization ratio chosen for the particular round in question. Following the aforementioned series of ratios of 1:10, 1:100, 1:500, AND 1:25000, the corresponding amounts of tester are 500ng, 50ng, 1ng, and 0.2ng. **It is also important to note that a different set of 12/24mer primers is used for each RDA round.**

To perform rounds 2-4, repeat the steps outlined above in sections I though P, making sure to use new primers and the amount of tester that is appropriate for the particular round in question. Always ligate 1µg of tester, then dilute the ligated stock to a concentration that is appropriate for the amount of tester needed.

In rounds three and four, it may be advantageous to increase the number of PCR cycles in the second run (after the first 7 cycles) to 25 cycles and 30 cycles, respectively. This will result in increased product yields for these rounds, where the amounts of tester are very low initially.

Upon completion of the RDA procedure, the differential expression of the cloned cDNAs must be verified by Northern blot analysis. However, prior to investing the considerable

time and effort required to isolate these cDNAs and insert them into plasmid vectors, is advisable to perform a “shotgun” Southern to provisionally verify that they are truly differentially expressed. The shotgun Southern can be performed quickly by collectively using the cDNA products of the RDA as probe templates to interrogate the two driver populations. This is done by making two Southern blots of the driver cDNAs and probing the blots with either labeled Red RDA product or labeled Green RDA product. If the RDA was successful, the Red probes should only hybridize to species in the Red driver, and the Green products should only hybridize to species in the Green driver. Following this “shotgun” Southern, the same strategy can be employed to perform a “shotgun” Northern using the RNAs corresponding to the original RNAs that were used in the RDA. Using this “shotgun” approach can conserve time and energy, as well as reducing the amount of RNA that is required to verify that the RDA products are, in fact, differentially expressed.

Reagents

- DPN II (New England Biolabs, MA)
- T4 DNA Ligase (New England Biolabs, MA
- Taq Polymerase (Perkin-Elmer,MA or Promega,WI)
  (Comes with 10X PCR Buffer and 25mM $MgCl_2$ Solution)

- dNTPs (New England Biolabs,MA or Promega,WI)

- Sephecryl S-300 (Pharmacia, NJ)
  alternatively PCR Select-II columns (5Prime-3Prime,CO)) can be used.

- Molecular Biology Grade Water (Eppindorf, NY or Promega,WI)

- Primers Synthesized by Genosys,TX

- Hybridization Buffer.
  30mM EPPS (N-(2-Hydroxyethyl)piperazine-N'-(3-propanesulfonic acid)) pH 8.0 at 25°C

  3mM EDTA Ethylenediaminetetraacetic acid
  pH 8.0 at 25°C
  (Store in small aliquots at -20°C)

- Tris-Cl/ EDTA (TE) Buffer
  10mM Tris-Cl
  1mM EDTA
  pH 7.5 at 25°C