

Automation of Fluorescent Differential Display With Digital Readout

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Summary

Since its invention in 1992, differential display (DD) has become the most commonly used technique for identifying differentially expressed genes because of its many advantages over competing technologies such as DNA microarray, serial analysis of gene expression (SAGE), and subtractive hybridization. Despite the great impact of the method on biomedical research, there has been a lack of automation of DD technology to increase its throughput and accuracy for systematic gene expression analysis. Most of previous DD work has taken a “shot-gun” approach of identifying one gene at a time, with a limited number of polymerase chain reaction (PCR) reactions set up manually, giving DD a low-tech and low-throughput image. We have optimized the DD process with a new platform that incorporates fluorescent digital readout, automated liquid handling, and large-format gels capable of running entire 96-well plates. The resulting streamlined fluorescent DD (FDD) technology offers an unprecedented accuracy, sensitivity, and throughput in comprehensive and quantitative analysis of gene expression. These major improvements will allow researchers to find differentially expressed genes of interest, both known and novel, quickly and easily.

Key Words: Fluorescent differential display; DD; FDD; differential gene expression; automation; differential display on automated sequencer.

1. Introduction

How can a single fertilized egg containing a complete set of genes unique to a species give rise to so many different cell types that will ultimately organize into the different tissues and organs that define each specific organism? This has been one of the most elusive questions in biology, because even complete sequencing of many genomes, from a few thousand basepairs for bacteria to

over 3 billion basepairs for human, has yet to provide enough clues to this mystery of life. Of the estimated 20,000–25,000 genes embedded in our genome, only a fraction of them, perhaps 10–15%, are “turned on” (expressed as mRNAs for protein synthesis) at any given time in each of our cells. Thus, interpretation of the genomic instructions in the post-genome era will have to rely, at least in large part, on tools that can allow us to determine when and where a gene is to be turned on or off in a cell as it divides, differentiates, and ages. Such tools are also important for the detection of when and where a seemingly precise interpretation of genomic instruction goes awry, which underlies many disease states such as cancer. Differential display (DD) technology (**1**) is one of the major tools that has already helped thousands of researchers all over the world interpret gene expression in their specific projects.

DD technology continues to be one of the most reliable methods for gene expression analysis available to biomedical researchers. Since its invention in 1992, the number of publications using DD has exploded to over 3600, easily outnumbering the publications using other competitive methodologies such as DNA microarrays (**2,3**), serial analysis of gene expression (SAGE) (**4**), and subtractive hybridization (**5**) (see **Table 1**). It is clear that the rapid and successful adoption of differential display has been largely attributed to the simplicity of the method. Simplicity ensures a higher probability of success and few artifactual differences caused by experimental errors. Essentially, starting from the RNA samples being compared, only two steps, reverse transcription and polymerase chain reaction (PCR), are needed before signals generated are analyzed on a gel matrix. No additional steps such as second-strand DNA synthesis, purification of cDNA, restriction enzyme digestion, adapter primer ligation, probe labeling/normalization, hybridization, or washing steps are required, because each of these steps could introduce and amplify errors or lead to the loss of mRNAs being detected.

DD takes advantage of three of the most simple, powerful, and commonly used molecular biological methods: reverse transcriptase (RT)-PCR, DNA sequencing gel electrophoresis, and cDNA cloning (**1,6**). The DD methodology, also referred to as DDRT-PCR or DD-PCR in PCR nomenclature (**7,8**), begins with total RNA being harvested from the cells/tissues of interest. A researcher will study at least two samples, but many more can be studied if the experiment suggests so. These samples will have morphological, genetic, or other experimental differences for which the researcher wishes to study the gene expression patterns, hoping to elucidate the root cause of the particular difference or specific genes that are affected by the experiment. Samples can be from any eukaryotic organism, including plants, fish, amphibians, reptiles, insects, yeast, fungi, and mammals. DD can be adapted for prokaryotic systems, but is more often used with eukaryotes.

Table 1
Impact of Major Technologies in Differential Gene Expression Analysis

Method	No. of citations ^a	Original publication ^b
Differential display	3606	Science (1992) 257 , 967–971
DNA microarrays	2296	Science (1995) 270 , 467–470
SAGE	1448	Science (1995) 270 , 484–487
Oligo arrays	742	Science (1996) 274 , 610–614

^aNumber of citations is the number of times the original publication has been cited by other papers, which reflects the number of times each technique has been used for publications.

^bSearch done with ISI Web of Knowledge Citation Search. Search conducted on January 19, 2005 at <http://isi15.isiknowledge.com/portal.cgi?DestApp=WOS&Func=Frame>.

The messenger RNAs (mRNAs) within the total RNA population are used as the templates for DD-PCR after first-strand cDNA synthesis by reverse transcription. The current methodology makes use of three “anchored” oligo-dT primers that target the poly-adenylation site of eukaryotic mRNA and have the form H-T₁₁M, where H is a *Hind*III restriction site (AAGCTT), T₁₁ is a string of 11 Ts (though the first two Ts come from the *Hind*III site), and M is G, C, or A (**9**). They are referred to as “anchor” primers because the non-T base after the string of 11 Ts enables the primer to be anchored to the same spot for each round of amplification, in contrast to standard oligo-dT primers that only contain a string of Ts and will anneal in multiple spots, creating a smear (*see* **Note 1**). The *Hind*III restriction site was added to the anchor primer design to make the primers longer and more efficient in annealing to the targeted poly-A site, as well as improving downstream applications such as cDNA cloning. Using the current anchor primer design, the cDNA populations are subsequently divided into three subpopulations that represent one-third of the potential mRNA expressed in the cell at any given time. Previous work indicated using anchor primers of the type T₁₁VN, where V can be A, G, or C and N can be any of the four nucleotides, as well as anchors of the type T₁₂MN, where M is a degenerate mixture of A, G or C, and N is any of the four nucleotides (**1**). Both of those primer designs result in larger subfractions of the mRNA population (12 for type T₁₁VN and 4 for T₁₂MN), which unnecessarily increases the amount of FDD-PCR reactions for the same level of gene coverage vs the H-T₁₁M primer design.

The next step in DD is the PCR-amplification of the cDNA subpopulations utilizing a combination of anchor primers (called H-T₁₁M) with a set of “arbitrary” primers that are random and short in length. The design of these arbitrary 13mers (H-AP primers) utilized in DD technology also includes a *Hind*III restriction site (AAGCTT) and a 7-basepair backbone of random base combinations. The *Hind*III restriction site is included in both the anchor and arbitrary

primers for more efficient primer annealing and easier downstream manipulation of the cDNA (9). The primers used in DD represent a random selection from over 16,000 (4^7) basepair combinations. Additionally, the length of an arbitrary primer is so designed that by probability each will recognize 50–100 mRNAs under a given PCR condition (10). As a result, mRNA 3' termini defined by any given pair of anchored-primer and arbitrary primer are amplified and displayed by denaturing polyacrylamide gel electrophoresis (PAGE). A mathematical model of estimated gene coverage utilizing various combinations of anchor and arbitrary primers was developed shortly after the advent of DD technology (10). This mathematical model indicated that approx 240 primer combinations (three anchor primers with 80 arbitrary primers) were needed to approach the level of estimated genome-wide screening for eukaryotes (approx 95%). However, a new mathematical model presented in the previous chapter of this book, predicts that more primer combinations are required to give that level of coverage; using 480 primer combinations (3 anchor primers with 160 arbitrary primers) would provide approx 93% coverage based on the new model.

DD was originally optimized with radioactivity using ^{35}S (1). ^{33}P labeling was then developed (9) for better sensitivity and resolution and has been the most commonly used for publications. However, fluorescent differential display (FDD) (see Fig. 1) was the next logical progression. In the development of FDD, it was crucial that the new platform have similar sensitivity to traditional DD with isotopic labeling, as well as other advantages that would make the platform a viable and improved alternative to the established DD methodology. FDD, optimized using fluorochrome-labeled anchor primers (generically called FH- T_{11}M) and higher dNTP concentrations in PCR, was shown to be essentially identical in both sensitivity and reproducibility to that of conventional DD (6) (see Fig. 2). Improvements such as elimination of radioactivity, digital data acquisition, and increased assay speed were goals that were successfully reached by the establishment of the FDD platform, representing a marked improvement over conventional DD.

After PCR amplification, gel electrophoresis is performed to separate the resulting PCR products by size. Reactions are run side by side so that the samples being compared are next to one another for each primer combination. Comparison of the cDNA patterns between or among relevant RNA samples reveals differences in the gene expression profile for each sample (see Fig. 3). Electrophoresis can be performed with denaturing polyacrylamide sequencing gels (1,11), nondenaturing polyacrylamide gels (7), or with agarose gels (12,13). Sequencing gels are the most commonly used method and are recommended here because they offer the best band resolution and allow for easy and efficient recovery of genes. In addition, their ability to accommodate a large number of reactions reduces the number of gels that must be run for FDD analysis.

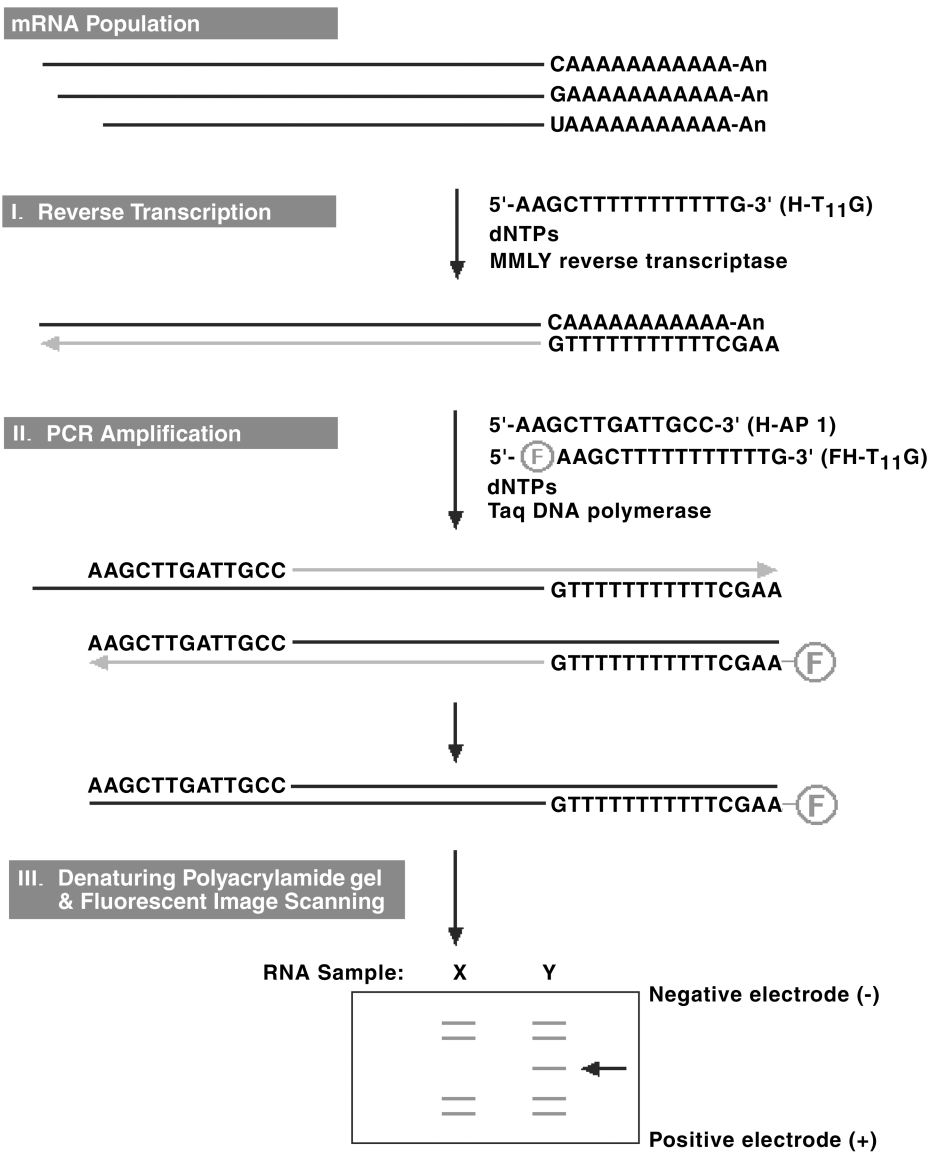
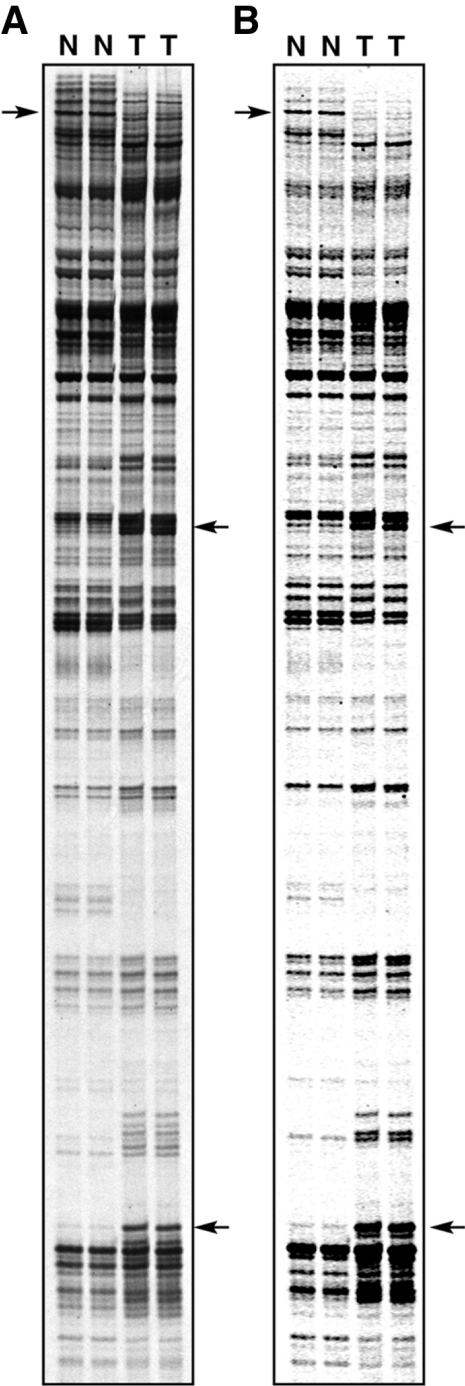


Fig. 1. Schematic representation of fluorescent mRNA differential display. Three fluorescently labeled one-base anchored oligo-dT primers with 5' *Hind*III sites are used in combination with a series of arbitrary 13mers (also containing 5' *Hind*III sites) to reverse transcribe and amplify the mRNAs from a cell.



Because the resulting cDNAs are fluorescently labeled, the use of a fluorescent imager scanner is required for this technology. Here the FMBIO[®] laser imager series (MiraiBio, Alameda, CA) is recommended for digital acquisition of the cDNA profiles. Although this is the recommended imager, other fluorescent scanners, such as the Typhoon[®] (Amersham Biosciences, Piscataway, NJ) and FLA-5000 (FUJIFILM Medical Systems, Stamford, CT) can also be used for FDD with similar sensitivity.

Another option for visualization of PCR reactions is to run samples on an automated sequencer. Our group has successfully used the Applied Biosystems ABI3100, a capillary array-based automated sequencer, for FDD band detection with several different fluorophores. These capillary electrophoresis (CE) machines have a laser at a fixed point and when a fluorescently labeled product passes the laser, a signal is detected. The results of FDD are seen as a series of spectral peaks for each lane, which can be compared to show differences in a very sensitive and reproducible way (*see Fig. 4*). The use of CE can dramatically cut down on the time and labor required for large-scale FDD screenings. However, the major drawback and bottleneck for using this technology with FDD is that, at this point, there is no way to retrieve bands from the CE results. One would still have to run a gel and detect bands using an alternate method. The most sophisticated attempt to solve this bottleneck was the development of a prototype computer-controlled CE system for positive band identification and retrieval by fraction collection by the Hitachi Japan group (*14*). But, to our knowledge, no further progress or commercialization has been made.

Upon completion of the gene expression profiles by gel electrophoresis, the next step is to begin characterization of the potential differentially expressed genes of interest. Bands are excised from the gel matrix and reamplified with the same primer combination as the original FDD-PCR and under the same reaction conditions. Generally, a PCR-product cloning step is recommended before differential gene confirmation and sequencing, but this is up to the preferences of the researcher. The PCR-TRAP[®] Cloning System (GenHunter Corporation, Nashville, TN) is recommended because it is designed specifically

Fig. 2. (*opposite page*) Comparison of radioactive and fluorescent differential display. DNA-free RNA from normal (N) and *ras* oncogene transformed (T) rat embryo fibroblasts were compared in duplicate by either conventional differential display with ³³P-labeled- α -dATP or FDD with fluorescein-labeled anchor primer under identical PCR conditions. The autoradiogram (A) and fluorescent images in grayscale (B) were compared in sensitivity and reproducibility as indicated. Reproducible differences are marked by arrows. The anchored primer, H-T₁₁G, was used in combination with arbitrary 13mer, H-AP29.

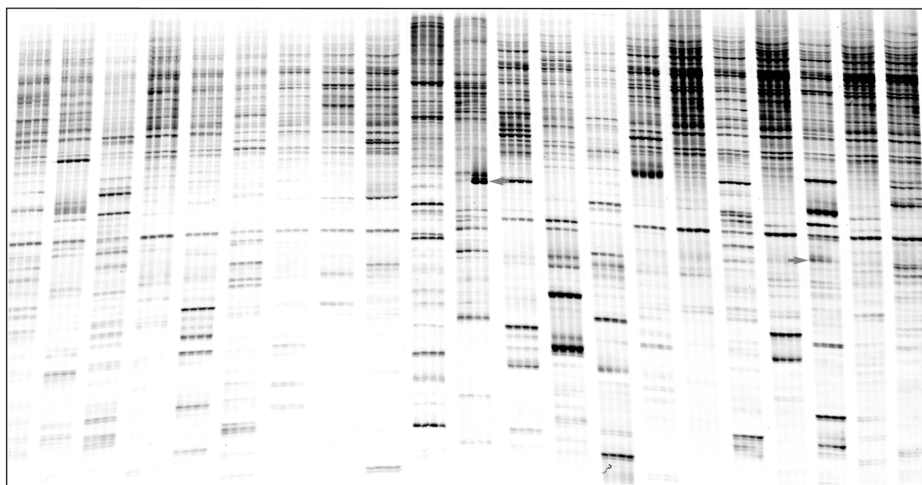


Fig. 3. Automated FDD result. Four RNA samples (before, and 6, 9, and 12 h after a drug treatment) were compared with 1 anchor primer in combination with 24 arbitrary primers (only 21 shown) using automation in liquid-handling, 132-lane electrophoresis unit, and digital acquisition of gel image. Grey arrows indicate reproducible differences worthy of pursuit.

for cloning DD bands and employs highly efficient positive-selection cloning. Because of the potential that more than one distinct cDNA is contained within an excised band, more than one colony should be screened for the correct size before it is characterized. Furthermore, if the screening results indicate that more than one cDNA is present in the colony population, each of the different cDNAs should then be further characterized.

Characterization of each potential gene includes sequencing of the cloned cDNAs of interest, with the results giving an indication of whether the cDNA is a known or unknown sequence. As with any differential gene expression technology, one has to be sure that the characterized sequences are actually differentially regulated, i.e., a “real difference,” and not a false positive. A variety of confirmation techniques, including Northern blot analysis, reverse Northern blot analysis, quantitative RT-PCR (qRT-PCR), or real-time PCR can be used. Although each has its own distinct advantages and disadvantages, Northern blot analysis is considered the gold standard for gene expression confirmation and is therefore recommended. Despite being labor-intensive, time-consuming, and requiring a significant amount of RNA, the Northern blot is by far the most accepted tool for confirmation. Northern blots have a distinct advantage over other confirmation methodologies in sensitivity, because both high- and low-level mRNA expression can be validated with this standard assay.

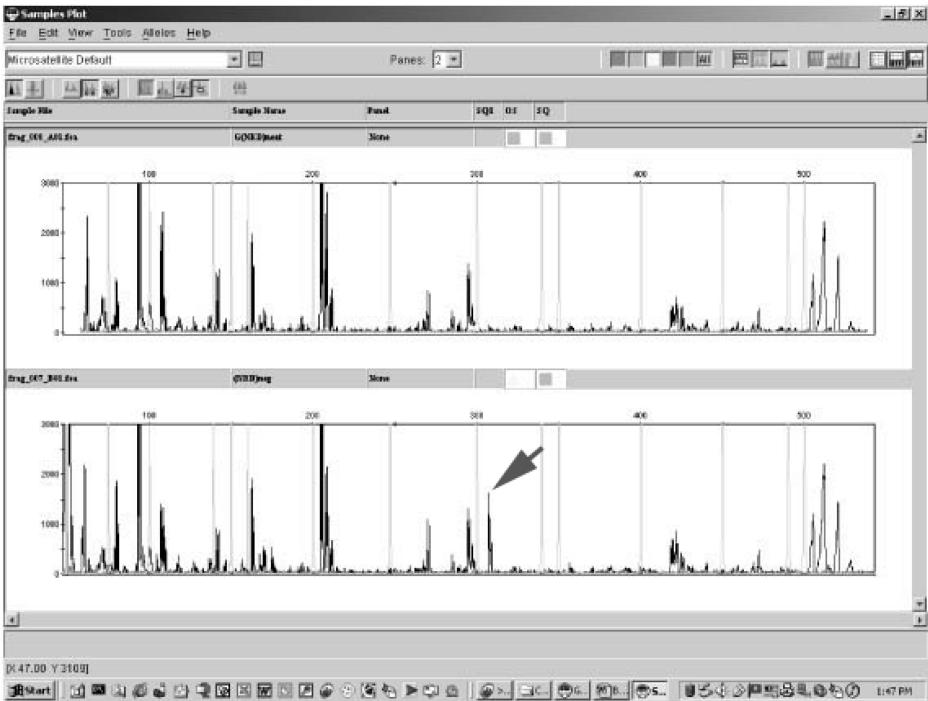


Fig. 4. Capillary electrophoresis of FDD reactions. RNA samples without (–) and with (+) p53 activation are compared by FDD and samples are run on ABI3100 Capillary Electrophoresis instrument. A candidate p53 target gene shows up regulation in the +p53 sample at approx 305 bp.

The optimized FDD technology is now able to compete with other gene expression tools such as DNA microarray technology because of improved high-throughput capabilities, while maintaining its inherent advantages over microarrays. Because the DD approach to differential gene expression analysis relies on randomly generated primers, no prior knowledge of the mRNA sequences is required, making the gene screening systematic, nonbiased, with the ability to find unknown genes. In addition, DD allows researchers to study more than two samples simultaneously, with only 10–20 μ g total RNA required for a “complete coverage.” Disadvantages of microarray technology as compared to FDD are reproducibility, probe sensitivity, nonlinearity in signal detection (15), probe cross-hybridization owing to homologous cDNA sequences (16), and data management (17). Depending on the amount of desired gene coverage, FDD methodology enables quicker results when compared to traditional isotopic DD or other DD-related technologies, yet ensures

more reliable results when compared to microarray or other competing, non-DD technologies. Combined with robotics and digital data analysis, FDD has been shown to be even more accurate and high throughput (6,18). Elimination of manual reaction set-up, through the use of a robotic liquid dispenser, not only ensures reproducibility by reduction of pipetting errors, but, in combination with the elimination of conventional DD autoradiography, also decreases the amount of time required for a differential gene expression screening. This technology allows researchers to quickly and easily find the truly differentially expressed genes in their project so they can spend their time and effort on the downstream functional characterizations, where some of those mysteries of life can be pieced together.

2. Materials

2.1. Total RNA Isolation

1. Phosphate-buffered saline (PBS).
2. RNA isolation reagent: a phenol-guanidinium monophasic solution such as RNeasy[®] (GenHunter, cat. nos. P501 to P503) is recommended.
3. Chloroform.
4. Polytron[™] Homogenizer for RNA extraction from tissue (Biospec Products Inc., Bartlesville, OK).
5. Diethyl pyrocarbonate-(DEPC)-treated water (GenHunter, cat. no. R105).
6. Isopropanol.
7. 100% ethanol.
8. 70% ethanol in DEPC-treated dH₂O.
9. 1.7 mL microfuge (Denville Scientific, Metuchen, NJ).

2.2. Removal of Genomic DNA From Total RNA

1. MessageClean[®] DNA Removal Kit (GenHunter, cat. no. M601) including RNase-free DNase I (10 U/ μ L), 10X reaction buffer (100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM MgCl₂, and 0.01% gelatin), 3 M sodium acetate, pH 5.5, DEPC-treated water, and RNA loading mix.
2. Agarose, ultraPure (Invitrogen, Carlsbad, CA).
3. Distilled water (double distilled and autoclaved).
4. Phenol/chloroform (3:1) solution, Tris saturated: 30 mL melted crystalline phenol, 10 mL chloroform, 10 mL Tris-HCl, pH 7.0.
5. 10X MOPS Buffer: 0.2 M MOPS, 0.05 M sodium acetate, 0.01 M ethylenediamine tetraacetic acid (EDTA), pH 6.5.
6. 12.3 M (37%) formaldehyde, pH >4.0.

2.3. Single-Strand cDNA Synthesis by Reverse Transcription

1. RNAspectra[™] Fluorescent Differential Display Kit (GenHunter, cat. nos. R501-R510 and F501-F510) including distilled water, 5X RT buffer (125 mM Tris-Cl,

- pH 8.3, 188 mM KCl, 7.5 mM MgCl₂, and 25 mM dithiol threonine [DTT]), deoxyribonucleoside triphosphate (dNTP) mix (FDD), oligo-dT anchor primers (H-T₁₁M, 2 μ M), and MMLV reverse transcriptase (100 U/ μ L).
2. 0.2 mL thin-walled PCR tube, RNase-free (GenHunter, catalog no. T101).
 3. Thermal cycler, GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA).

2.4. FDD-PCR

1. RNAspectra™ Fluorescent Differential Display Kit (GenHunter, cat. nos. R501-R510 and F501-F510) including distilled water, 10X PCR buffer (100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM MgCl₂, and 0.01% gelatin), FDD dNTP mix, fluorescent anchor primers (R-H-T₁₁M or F-H-T₁₁M), and arbitrary primers (H-AP, 2 μ M).
2. *Taq* DNA polymerase (Qiagen, Valencia, CA, cat. no. 201207).
3. 0.2 mL thin-walled PCR tube, RNase-free (GenHunter) or 96-well PCR plates (Thermo-Fast® 96 Detection Plate, ABgene Inc., Rochester, NY, cat. no. AB-1100).
4. Liquid-handling robot. GenHunter uses the Biomek 2000 (Beckman Coulter Inc., Fullerton, CA).

2.5. Gel Electrophoresis

1. Gel apparatus with low-fluorescent (borosilicate) glass plates such as Horizontal or Vertical FDD Electrophoresis Systems (GenHunter, cat. no. SA101 or SA201).
2. 5 M KOH.
3. 50% ethanol (EtOH).
4. Sigmacote® (Sigma, St. Louis, MO) or similar product.
5. 6% denaturing gel solution such as Sequagel 6 Ready-To-Use 6% Sequencing Gel® (National Diagnostics, Atlanta, GA, cat. no. EC-836)
6. 10X TBE: 0.89 M Tris-borate, pH 8.3, 20 mM disodium EDTA (Na₂EDTA).
7. 10% ammonium persulfate (APS).
8. FDD Loading Dye from RNAspectra Kit (GenHunter, cat. no. F201): 99% formamide, 1 mM EDTA, pH 8.0, 0.009% xylene cyanole FF, and 0.009%.
9. Fluorescent Laser Scanner. The FMBIO® II or III Series (MiraiBio, Alameda, CA) is recommended.
10. UV-transparent plastic wrap. Standard Glad® Cling Wrap (Glad Products Company, Oakland, CA) or Saran Wrap work well.
11. FDD locator dye (GenHunter, cat. no. F202 and R202).

2.6. Reamplification of Selected Differentially Expressed Bands

1. Distilled water.
2. 3 M sodium acetate, pH 5.5, from GenHunter MessageClean Kit.
3. Glycogen, 10 mg/mL (GenHunter, cat. no. S301).

4. 100% ethanol.
5. 85% ethanol.
6. Unlabeled anchor primers (H-T₁₁G, H-T₁₁A, H-T₁₁C; 2 μ M, from GenHunter RNAspectra Kit or cat. no H101-S).
7. Arbitrary primers (H-AP1 to H-AP80, 2 μ M, from GenHunter RNAspectra Kit or cat. nos. H-AP1 to H-AP80).
8. *Taq* DNA polymerase (Qiagen, cat. no. 201207).
9. dNTP Mix (FDD) from RNAspectra Kit.
10. 10X PCR buffer (GenHunter, cat. no. S201): 100 mM Tris-Cl, pH 8.4, 500 mM KCl, 15 mM MgCl₂, and 0.01% gelatin.
11. Agarose.
12. 10X Agarose DNA loading dye: 40% sucrose, 0.1% bromophenol blue, 0.1% xylene cyanole FF, 2.5 mM in distilled water.
13. 0.2 mL thin-walled PCR tube, RNase (GenHunter).

2.7. Cloning of Reamplified PCR Products

1. PCR-TRAP Cloning System (GenHunter, cat. no. P404) including insert-ready PCR-TRAP cloning vector, T4 DNA ligase (200 U/ μ L), distilled water, 10X ligase buffer (500 mM Tris-HCl, pH 7.8, 100 mM MgCl₂, 100 mM DTT, 10 mM ATP, 500 μ g/mL bovine serum albumin [BSA]), Lgh/Rgh primers (2 μ M), Colony Lysis Buffer (1X TE with 0.1% Tween-20), 10X PCR buffer, dNTP 250 μ M, tetracycline (20 mg/mL), and GH competent cells.
2. Luria Bertani (LB) media. Make 1 L LB with 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, and up to 1 L with dH₂O.
3. LB-Agar-TET plates. Make 1 L LB-Agar-TET plates with 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 9 g NaCl, 15 g Bacto-agar, and up to 1 L with dH₂O. Autoclave 60 min then and add 1 mL tetracycline (20 mg/mL) when liquid cools to approx 50°C. Pour plates.
4. Bacterial polystyrene Petri dish.
5. 0.2 mL thin-walled PCR tube, RNase-free (GenHunter).
6. 1.7 mL microfuge tubes (Denville Scientific).
7. QIAEX™ II Gel Extraction Kit (Qiagen, cat. no. 20021).

2.8. Sequencing of Cloned PCR Products

1. AidSeq Primer Set C (GenHunter, cat. no. P203): includes Lseq and Rseq primers.
2. QIAquick PCR Purification Kit (Qiagen, cat. no. 28106).

2.9. Confirmation of Differential Gene Expression by Northern Blot

1. Lgh/Rgh Primers (2 μ M) from PCR-TRAP Cloning System or alone (GenHunter, cat. nos. L201 and L202).
2. *Taq* DNA polymerase.
3. dNTP 250 μ M from PCR-TRAP Cloning System or alone (GenHunter, cat. no. S501).

4. 10X PCR buffer from PCR-TRAP Cloning System or alone (GenHunter, cat. no. S201).
5. Colony lysis buffer from PCR-TRAP Cloning System or alone (GenHunter, cat. no. L102).
6. HotPrime[®] DNA labeling kit (GenHunter, cat. no. H501) including Klenow DNA polymerase (1 U/ μ L), 10X labeling buffer, dNTP (-dATP) or dNTP (-dCTP) (500 μ M), stop buffer, and distilled water.
7. QIAEX[™] II Gel Extraction Kit.
8. Agarose.
9. 10X MOPS buffer.
10. 12.3 M (37%) formaldehyde, pH >4.0.
11. Distilled water.
12. Lock-top microfuge (USA Scientific, Ocala, FL, cat. no. 1415-5100).
13. α -[³²P] dATP (3000 curies/mmol) (PerkinElmer Life Sciences, Boston, MA, cat. no. BLU512H).
14. Sephadex G50 column (Roche Applied Science, Indianapolis, IN, cat. no. 1814419).
15. Salmon sperm DNA (10 mg/mL) (GenHunter, cat. no. ML2).
16. Nylon membrane: Nytran SuperCharge Nylon Transfer Membrane (Schleicher and Schuell, Keene, NH, cat. no. 10 416 216).
17. Paper towels.
18. UV-transparent plastic wrap.
19. Single-emulsion scientific imaging film. Kodak Biomax MS (Kodak-Eastman, Rochester, NY, cat. no. 8715187) is recommended.
20. 20X saline-sodium citrate (SSC): 3 M NaCl, 0.3 M trisodium citrate. Adjust pH to 7.0 with 1 M HCl.
21. Formamide prehybridization/hybridization solution (GenHunter, cat. no. ML1).
If preparing in the lab, use the following protocol for 500 mL:

20X Saline-sodium phosphate-EDTA (SSPE)	125 mL
50X Denhardt's Solution	50 mL
20% Sodium dodecyl sulfate (SDS)	2.5 mL
Formamide	250 mL
Distilled water	up to 500 mL

Mix well, aliquot into smaller volumes, and store at -20°C until use.

To make 20X SSPE: 3 M NaCl, 0.1 M NaH₂PO₄ (dibasic), 0.01 M EDTA.

To make Denhardt's Solution, 50 mL:

Ficoll	0.5 g
Polyvinylpyrrolidone	0.5 g
BSA (Pentax Fraction V)	0.5 g
Distilled water	Up to 50 mL

22. 1X SSC, 0.1% SDS (w/v).
23. 0.25X SSC, 0.1% SDS (w/v).

3. Methods

3.1. Total RNA Isolation

Although FDD takes advantage of the poly-adenylation (poly-A+) site of eukaryotic mRNA, total RNA is preferred over poly-A+ RNA (mRNA) for several reasons. These reasons include the overall ease of purification, the ability to verify RNA integrity, and the cleaner background signal (*see Note 2*). To this end, total RNA is suggested for FDD analysis.

If one is planning to do a 240-primer combination screening with FDD, approx 12 µg of “cleaned” total RNA is required. The term “cleaned” refers to being clean of DNA achieved by DNase I treatment described in **Subheading 3.2**. Generally, 50–80% of the beginning amount of total RNA can be retrieved after cleaning. In addition, it is important to make sure there is plenty of RNA left over for whatever confirmation step is chosen. To ensure there is enough RNA for all steps, it is suggested to isolate approx 50 µg of total RNA. The amount of total RNA that can be isolated from a sample can vary widely depending on the tissue/cell type, procedure used, organism, and proficiency at that particular procedure. However, using a reagent based on the standard phenol/guanidine thiocyanate technique such as RNeasy[®], one can achieve an average yield of 50 µg of total RNA from 25 mg of tissue or 2.5×10^6 cells (*see Note 3*).

3.1.1. RNA Extraction From Various Sources

3.1.1.1. EXTRACTION OF RNA FROM TISSUE CULTURES

1. If using regular “attached” cells, pour off medium. Set the plate on ice. If cells are in suspension, spin down cells, remove the medium, then move on to **step 4**.
2. Rinse cells with 10–20 mL of cold PBS.
3. Pour off rinsed PBS and remove the residual PBS with a 1000 µL pipet (*see Note 4*).
4. Add 2 mL of RNeasy RNA isolation reagent per 100- to 150-mm plate to lyse the cells. Spread the solution by shaking the plate. This volume is sufficient for 1–10 million cells.
5. Let sit on ice for 10 min.
6. Pipet the lysate into two labeled 1.5-mL microfuge tubes.

3.1.1.2. EXTRACTION OF RNA FROM TISSUES

1. Add at least 2 mL of RNeasy RNA isolation reagent to the tissue in a 50 mL conical tube on ice. Ideally, the volume ratio of RNA isolation reagent to tissue should be at least 10:1.
2. Homogenize the tissue with a Polytron[™] Homogenizer until the tissue is dispersed.
3. Let sit on ice for 10 min.
4. Transfer 1 mL aliquots of the lysate into labeled 1.5 mL centrifuge tubes.

3.1.1.3. EXTRACTION OF RNA FROM BLOOD

1. Spin down blood products and remove the plasma.
2. Follow the instructions in **Subheading 3.1.1.2.**

3.1.2. RNA Purification

1. Add 150 μL of chloroform/mL of lysate. Vortex for 10 s. Protocol can be stopped here by placing the lysates at -80°C .
2. Centrifuge the tubes at 4°C with maximum speed for 10 min (*see Note 5*).
3. Carefully remove the upper phase (*see Note 6*) into a clean, labeled 1.5-mL centrifuge tube. If RNA is being isolated from tissues, a second extraction is generally recommended to remove any RNases (*see Note 7*).
4. Add an equal volume of isopropanol. Mix vigorously or vortex for 30 s. Let sit on ice for 10 min.
5. Centrifuge for 10 min at 4°C with maximum speed.
6. Rinse the RNA pellet with 1 mL of cold 70% ethanol (in DEPC-treated water). Centrifuge 2 min at 4°C with maximum speed.
7. Remove the ethanol. Spin briefly and remove the residual wash solution with a pipet.
8. Resuspend the RNA in DEPC-treated water. The amount used for resuspension will depend on the amount of RNA isolated, but the RNA should be at a concentration greater than 1 $\mu\text{g}/\mu\text{L}$, so adjust accordingly. Do not use SDS in resuspension if using RNA for any PCR application.
9. Measure the concentration by taking 1 μL of the RNA (using P10 pipet) and diluting to 1 mL of water (a 1:1000 dilution). Read at 260 nm. 1 $\text{OD}_{260} = 40 \mu\text{g}$
10. Move on to next steps and store RNA that will not be “cleaned” in aliquots at -80°C until next use.

3.2. Removal of Genomic DNA From Total RNA

For the purposes of FDD gene expression analysis, as well as any other RNA-based gene expression technologies, contaminating genomic DNA must be removed before single-strand cDNA synthesis by reverse transcription and subsequent PCR reactions. If left unchecked, any primers with matching sequence to the contaminating DNA will anneal during the FDD-PCR reactions, causing amplification of DNA sequences and leading to a higher false-positive rate. Therefore, the following protocol is one of the most important procedures in preventing irregularities or artifacts during the FDD-PCR reactions by removal of the contaminating genomic DNA. It is important to note that one will typically retrieve 50–80% of the total RNA put into the reaction, so the amount to be cleaned must be adjusted to the amount needed for FDD.

3.2.1. DNase I Digestion of Total RNA

1. If necessary, dilute desired amount of RNA to be digested (maximum of 50 μg) with DEPC-treated water to a volume of 50 μL .

2. In a 1.5-mL centrifuge tube, add the following *in order* (total reaction volume is 56.7 μL):

Total RNA (10–50 μg)	50 μL
10X Reaction buffer	5.7 μL
DNase I (10 U/ μL)	1.0 μL

3. Mix gently and incubate at 37°C for 30 min (*see Note 8*).

3.2.2. Extraction and Ethanol Precipitation of DNA-Free RNA

1. Prepare phenol/chloroform solution (*see Note 9*) by melting crystalline phenol at 65°C.
2. Add 30 mL melted phenol to 10 mL chloroform and mix well.
3. Add 10 mL Tris-HCl, pH 7.0 and mix well. Allow saturation phase to form before using.
4. Add 40 μL of phenol/chloroform solution to each DNase I reaction (*see Note 10*). Vortex for 30 s.
5. Let sit on ice for 10 min.
6. Centrifuge with maximum speed for 5 min at 4°C.
7. Collect upper phase (*see Note 6*) and place in a clean, labeled 1.5-mL microfuge tube.
8. Add 5 μL 3 M sodium acetate and 200 μL 100% ethanol. Mix well.
9. Let sit at least 1 h at –80°C. Overnight to a few days at –80°C is fine.
10. Centrifuge at 4°C for 10 min with maximum speed to pellet RNA.
11. Carefully remove the supernatant and rinse the RNA pellet with 0.5 mL of 70% ethanol (in DEPC-treated water). Do not disturb the pellet.
12. Centrifuge for 5 min at 4°C with maximum speed and remove supernatant. Centrifuge again briefly, removing the residual liquid without disturbing the RNA pellet.
13. Resuspend the RNA in 10–20 μL of DEPC-treated water.

3.2.3. RNA Quantification and Integrity Verification

After cleaning, it is crucial to be able to determine both the quantity and quality of the RNA retrieved. The amount can easily be quantified by OD₂₆₀. The quality/integrity of the RNA is determined most accurately by running the RNA on an “RNA gel” and looking for the appearance of sharp ribosomal RNA bands.

1. Quantitate the RNA amount by OD₂₆₀ after 1:1000 dilution of the DNA-free RNA sample with distilled water.
2. Prepare an “RNA gel” (denaturing formaldehyde agarose gel with MOPS and formaldehyde) by the following protocol:
 - a. Add the following to a microwave-safe container:

10X MOPS	10 mL
Agarose	1–1.5 g
Distilled water	83 mL

- b. Microwave for approx 3 min or until agarose is melted.
 - c. Let agarose cool to at least 50°C (barely touchable by hand).
 - d. Add 7 mL of a 12.3 M (37%) formaldehyde solution. Gently mix.
 - e. Pour into prepared gel casting plate and add gel comb.
 - f. Running buffer (1 L) is made by diluting 100 mL of 10X MOPS with 900 mL of distilled water to a 1X concentration. Cover agarose gel with running buffer.
3. Check the integrity of the RNA (*see Note 11*) by resolving 2–3 µg of both pre-DNase and post-DNase RNA samples on a 7% formaldehyde agarose gel with RNA Loading Mix by the following protocol:
 - a. Add 1–10 µL (2–3 µg) of RNA to 20 µL RNA Loading Mix in a labeled 1.5-mL microfuge tube. Mix well.
 - b. Incubate at 65°C for 10 min.
 - c. Centrifuge sample briefly to collect condensation.
 - d. Put samples on ice for 5 min.
 - e. Load entire amount onto RNA gel.
 - f. Run at 50–60 V for approx 45 min or until resolution of the ribosomal subunits is achieved.

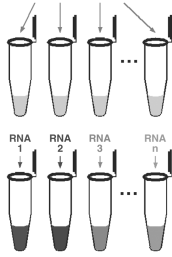
3.3. Single-Strand cDNA Synthesis by Reverse Transcription

Generally, two RT reactions are done per sample (called “in-duplicate”) to ensure reproducibility and as a way of reducing any false positives. It is recommended to set up separate RT core mixes for each individual H-T₁₁M in 200 µL volume RT reactions if 240 primer combinations will be performed. Therefore, if two samples are being studied, set up four 200 µL RT reactions for H-T₁₁G, four 200 µL RT reactions for H-T₁₁A, and four 200 µL RT reactions for H-T₁₁C. If smaller or larger numbers of primer combinations are chosen, adjust accordingly.

1. Dilute 40 µL of each RNA sample to a final concentration of 0.1 µg/µL with DEPC-treated water and mix thoroughly. Place on ice.
2. For an RT core mix with two samples in-duplicate for one H-T₁₁M primer (H-T₁₁G will be shown here), add the following:

376 µL distilled water
160 µL 5X RT buffer
64 µL FDD dNTP mix
80 µL H-T₁₁G primer
680 µL total volume
Mix well.

3. Divide the above 680 µL evenly into four tubes labeled with sample name (for example: RTG-1a, RTG-1b, RTG-2a, RTG-2b), aliquoting 170 µL per tube (*see Fig. 5* for step-by-step schematic of RT and FDD-PCR setup).



a) Add 170 μL RT Core Mix G to all

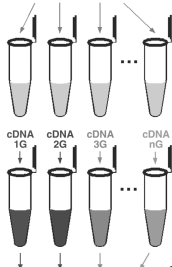
b) Add 20 μL of different total RNAs

c) Begin RT Reaction. After 10 min. at 37°C, add 10 μL MMLV RT to each reaction.

II. DD-PCR



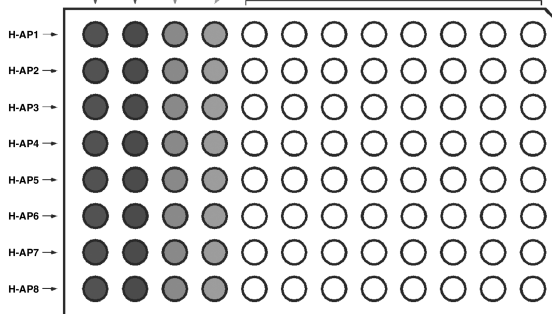
a) Add 1896 μL FDD Core Mix G & 24 μL Taq to a tube



b) Add 480 μL of FDD Core Mix G/Taq Mixture to all

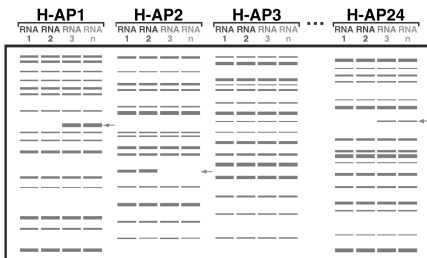
c) Add 60 μL of corresponding cDNA

Additional wells are used for additional reactions with H-AP arbitrary primers 9-24



d) Add 18 μL of cDNA/FDD Core Mix/Taq Mixture to each of the 8 wells down & 2 μL of each H-AP Arbitrary primer to each of the 4 wells across. Repeat for H-AP primers 9 to 24

III. Electrophoresis



a) Run out all samples on a 6% denaturing polyacrylamide gel

b) Cut out any bands of interest for reamplification, cloning, and confirmation.

4. Add 20 μL of corresponding total RNA (0.1 $\mu\text{g}/\mu\text{L}$, freshly diluted, *see* **Note 12**) to each tube. For example, add 20 μL of RNA 1 to each of tubes RTG-1a and RTG-1b followed by 20 μL of RNA 2 to each of tubes RTG-2a and RTG-2b. Mix each tube well.
5. Program the thermal cycler to: 65°C for 5 min, 37°C for 60 min, 75°C for 5 min, 4°C soak (*see* **Note 13**).
6. Place tubes on thermal cycler and begin program.
7. After the tubes have been at 37°C for 10 min, pause the thermal cycler and add 10 μL of MMLV reverse transcriptase to each tube. Quickly mix well by finger-tipping or pipetting up and down before continuing incubation program.
8. At the end of the reverse transcription, spin the tube briefly at maximum speed to collect condensation. Set the tubes on ice or store at -20°C for later use.
9. Repeat **steps 1–8** for H-T₁₁A and H-T₁₁C primers.

3.4. FDD-PCR

This protocol is designed for 240 primer combinations in duplicate per sample using three fluorescent dye-labeled anchor primers (FH-T₁₁M) and 80 upstream arbitrary primers (H-AP). This would yield approx 74% coverage of all possible genes. For a complete, genome-wide screening, 480 primer combinations or more must be completed per sample. It is ideal to set up PCR reactions in 96- or 384-well PCR plates using a robot to ensure reproducibility and increase throughput. Depending on the number of samples and the plate being used, one may be able to combine more or less than 24 primer combinations into one experiment. However, for simplicity, a 24-primer combination experiment with one anchor primer and two RNA samples in duplicate using a 96-well plate will be discussed. Therefore, this protocol will need to be repeated 10 times using varying anchor-arbitrary primer combinations.

1. A separate FDD-PCR core mix for each individual FH-T₁₁M primer is made. Here, a core mix for all 80 H-AP primers for FH-T₁₁G primer is shown. This will be called “FDD Core Mix G.”

4080 μL distilled water
800 μL 10X PCR buffer

Fig. 5. (*opposite page*) Schematic for reverse transcription (RT) and fluorescent differential display (FDD) reaction setup. This schematic shows individual steps involved and quantities required for standard RT and fluorescent differential display FDD reaction setups. These numbers are based on comparing two samples in duplicate (or four samples not in duplicate) with FH-T₁₁M anchor primer in combination with 24 H-AP arbitrary primers. These steps would be repeated 10 times until all 240-primer combinations (three anchor primers and 80 arbitrary primers) have been completed.

640 μ L dNTP Mix (FDD)

800 μ L FH-T₁₁G primer

6320 μ L total volume

Mix well.

2. Aliquot 1896 μ L of FDD Core Mix G into three separate tubes labeled “FDD Core Mix G” (see **Fig. 5** for step-by-step schematic of RT and FDD-PCR setup). Aliquot the remaining amount into a fourth tube labeled “FDD Core Mix G-remainder” (approx 632 μ L).
3. To one of the tubes labeled “FDD Core Mix G,” add 24 μ L *Taq* DNA polymerase. Mix well. Freeze the other three tubes aliquoted in **step 2** at -80°C for later PCR reactions.
4. Aliquot 480 μ L of “FDD Core Mix G/*Taq*” mixture to four separate tubes labeled corresponding to the RT reactions. For example, use FDDG-1a, FDDG-1b, FDDG-2a, FDDG-2b.
5. Add 60 μ L of corresponding cDNA from RT to each of the four tubes. For example, 60 μ L of RTG-1a tube would go into the tube labeled FDDG-1a. Mix well.
6. Using either a robot or by hand, add 2 μ L of H-AP primers 1–24 to corresponding wells of 96-well plate (see **Fig. 5**).
7. Using either a robot or by hand, add 18 μ L of corresponding FDD Core Mixes to corresponding wells of 96-well plate (see **Fig. 5**).
8. Total reaction volume will be 20 μ L. Add 25 μ L of mineral oil if needed.
9. Program the thermal cycler to:
 - 94 $^{\circ}\text{C}$ for 15 s (see **Note 14**)
 - 40 $^{\circ}\text{C}$ for 2 min
 - 72 $^{\circ}\text{C}$ for 60 s
 - for 40 cycles
 - 72 $^{\circ}\text{C}$ for 5 min
 - 4 $^{\circ}\text{C}$ soak.
10. Put 96-well plate on thermal cycler and begin program. Once completed, store reactions at -20°C in the dark.
11. **Steps 3–10** will then be repeated for H-AP primers 25–48 and 49–72.
12. The same process will then be done for H-AP primers 73–80 as follows (see **Note 15**):
 - a. Add 8 μ L *Taq* DNA polymerase to the 632 μ L of “FDD Core Mix G-remainder.” Mix well.
 - b. Aliquot 160 μ L of that mixture to four separate tubes labeled the same as **step 4**.
 - c. Add 20 μ L of cDNA from RT to each of the four tubes corresponding cDNA as in **step 5**. Mix well.
 - d. Using either a robot or by hand, add 2 μ L of H-AP primers 73–80 to corresponding wells of 96-well plate.

- e. Using either a robot or by hand, add 18 μL of corresponding FDD Core Mixes to corresponding wells of 96-well plate.
- f. Follow **steps 8–10**.

13. Repeat **steps 1–12** for FH-T₁₁A and FH-T₁₁C primers.

3.5. Gel Electrophoresis

Because performing a large-scale FDD experiment requires many hundreds of PCR reactions (960 in the previously mentioned experiments), one of the areas for improvement in making it more high-throughput is in the gels themselves. Using a gel apparatus with many lanes can speed up this process tremendously. One system that has been successfully used is the Horizontal FDD Electrophoresis System with 132 lanes and the “Microtrough System” containing grooved glass plates. This allows one to load at least one entire 96-well plate on one gel. In addition, the Microtrough System allows the researcher to use standard 10 μL pipet tips for sample loading instead of the difficult-to-use flat gel-loading tips required by standard sequencing apparatuses. Hand position during loading is more stable and relaxed with this system.

A multichannel pipettor for gel loading has also been tried. Matrix Technologies (Hudson, NH) manufactures several pipettors with width-expandable channels called “Matrix Equalizers.” The 8-channel Matrix Equalizer 384 with 0.5–12.5 μL volume range works fairly well. These pipettors have tips that move independently and can be spaced anywhere from 4.5 to 14.15 mm apart. For the gel loading, the tips were spaced at 9 mm for liquid uptake from a 96-well plate and then collapsed together to 4.5 mm for gel loading. However, this 4.5 mm distance only allows 87 lanes per gel, not enough to load an entire 96-well plate. A pipettor that could contract to 3 mm for gel loading would be ideal, but so far Matrix has not manufactured this. Therefore, using one of these pipettors has trade-offs: although it decreases the time required for gel loading and the chance of incorrect loading, fewer reactions can be run on the same gel. The other option is to load the PCR reactions using the Matrix pipettor at the 6 mm distance, loading every other well, but this requires re-configuration of the reaction setup.

For the aforementioned experiments that have 960 PCR reactions on 10 96-well plates, it is recommended to run 10 separate gels, each with one 96-well plate. One to two gels can generally be run per day, requiring 5–10 d to run all 10 gels.

For ease of use, the Sequagel 6 Ready-To-Use 6% Sequencing Gel is recommended for denaturing gel electrophoresis. However, a general protocol is given here for the 6% denaturing polyacrylamide gel that is recommended for resolution of cDNA profiles.

1. Thoroughly clean both sides of glass plates to be used with warm water and soap, ensuring that there is no previous gel debris or streaks (*see Note 16*). Be sure to rinse thoroughly afterward because soap residue may cause problems. KOH can be used occasionally for this purpose to strip off hard-to-clean residue.
2. Further clean the glass plates by wiping with a 50% ethanol (EtOH) solution. Make sure plates are completely dry.
3. Coat the interior surface of one of the plates (usually the notched one) with 500 μL Sigmacote or similar product using a Kim-Wipe to smoothly spread over the surface. Let dry for 1 min. This coating step allows the gel to preferentially stick to the noncoated plate upon separation of plates for band-cutting after running the gel.
4. Use 60 mL of the gel mixture for a $45 \times 28 \times 0.04$ cm gel.
5. Add 0.5 mL of 10% APS solution and mix thoroughly.
6. Pour gel into sequencing gel cast and let polymerize 1–2 h or overnight (*see Note 17*).
7. After polymerization, load the glass plates into the sequencing apparatus and add 1X TBE buffer to upper and lower buffer chambers.
8. Flush the urea from the gel wells and pre-run the sequencing gel in 1X TBE buffer for 30 min.
9. Add 3.5 μL of each FDD-PCR reaction with 2 μL of FDD loading dye. Alternatively, an appropriate ratio of loading dye (8 μL for 20 μL PCR reactions) can be added directly to the PCR reaction if the reactions will only be used for running gels. Incubate at 80°C for 2 min immediately before loading onto the gel. This step is to denature the DNA samples before gel loading.
10. After heat-denaturation, put samples on ice for 1–2 min.
11. Load maximum amount of sample (usually 3–4 μL) into wells. It is crucial that all urea be removed from the wells before loading samples (*see Note 18*). For best results, load 4–6 lanes and then stop briefly to reflush wells to remove urea. Load in appropriate groups, usually by primer combination.
12. Electrophoresis for 1.5–3 h at 60 W constant power (voltage not to exceed 2000 V) until the xylene cyanole dye (the slower moving dye) reaches the bottom of the gel. In a 6% gel, the xylene cyanole will co-migrate with DNA of approx 106 bp as a reference point. If voltage exceeds 2000, lower the wattage. Gel should be kept in the dark while running to prevent photo-bleaching of samples (*see Note 19*) either by using a dark room or covering the gel apparatus with a cardboard box.
13. Turn off power supply and remove the plates from the gel apparatus. Take off gel tape and remove spacers and comb (*see Note 20*). Clean the outside of the glass plates very well with warm water and 50% ethanol to remove any residue from gel or tape. Thorough cleaning is required to reduce background signal (*see Note 16*).
14. Scan the gel on a fluorescence imager with an appropriate filter, following the manufacturer's instructions based on the particular fluorophore being used.

3.6. Reamplification of Selected Differentially Expressed Bands

Assuming differentially expressed bands of interest are seen, those bands should be excised from the gel. Following excision, the cDNAs will be reamplified using the same anchor-arbitrary primer combinations and reaction conditions as the initial FDD-PCR reactions. The reamplification products can then be cloned and sequenced for further characterization.

1. Separate the glass plates by taking off the notched/smaller glass plate (*see Note 21*) leaving the gel attached to the unnotched/larger plate.
2. Place a layer of UV-transparent plastic wrap on top of the gel. This prevents contamination of the gel as well as making gel cutting easier.
3. Spot 0.5 μL of FDD locator dye at the upper and lower corners of the gel to allow orientation of the picture with the gel. The FDD locator dye, with its combination of fluorescent and visible dyes, can be used to easily align the gel with the printed template for band excision.
4. Rescan the gel with the gel facing up.
5. Print a real-size image on appropriately sized paper (*see Note 22*) using a quality ink jet or laser printer. This printed image will be used as the template to excise differentially expressed cDNAs.
6. Choose and label any bands to be cut (*see Note 23*). A logical band-naming nomenclature should be used such as RN-G-1A (RN = researcher name; G = FH-T₁₁G anchor primer; 1 = H-AP1 arbitrary primer; A = top differentially expressed band in lane).
7. Place the printout on the table top and lay the glass plate on top of it. Orient the plate so that the spots on the printout match up with those on the gel.
8. Excise each band with razor or other sharp utensil and place into a 1.5-mL microfuge tube labeled with the corresponding band name.
9. For each band being reamplified, add 100 μL of distilled water to the tube containing the corresponding gel slice.
10. Let soak for 10 min at room temperature.
11. Boil the tightly closed tube (with parafilm or lock-top tube) for 15 min to elute the cDNA from the gel slice.
12. Spin for 2 min at maximum speed to collect condensation and pellet the gel.
13. Transfer supernatant to a fresh 1.5-mL microfuge tube labeled tube. Discard tube with gel slice. Add 10 μL of 3 M sodium acetate, 5 μL of glycogen, and 450 μL of 100% ethanol per tube. Let sit for at least 30 min on dry ice or in a -80°C freezer.
14. Spin for 10 min 4°C at maximum speed to pellet the DNA. Remove the supernatant and rinse the pellet with 200 μL of ice-cold 85% ethanol. Spin briefly and remove the residual ethanol.
15. Dissolve the pellet in 10 μL of dH_2O .
16. Make a reamplification core mix for each of the anchor primers that is large enough to reamplify all FDD bands with that particular anchor primer:

a. A standard reamplification reaction will contain:

Distilled water	23.3 μ L
10X PCR buffer	4.0 μ L
dNTP mix (FDD)	0.3 μ L
H-AP primer (2 μ M)	4.0 μ L
H-T ₁₁ M (2 μ M) (see Note 24)	4.0 μ L
cDNA template	4.0 μ L
Taq DNA polymerase	0.4 μ L
<hr/>	
40 μ L total volume	

- b. Determine how many bands of each anchor primer will be reamplified. Multiply each of these by 10% to give a cushion for any pipetting inaccuracies. The number of bands for H-T₁₁G \times 10% = g; the number of bands for H-T₁₁A \times 10% = a; and the number of bands for H-T₁₁C \times 10% = c.
- c. Make a Reamplification Core Mix for each H-T₁₁M by multiplying the numbers for a “Standard Reamplification Reaction” by g, a, and c accordingly. However, for the core mixes, the H-AP primers and cDNA templates will not be added, because these will vary with each band. Make the core mix as follows:

Distilled water	23.3 μ L \times g, a, or c
10X PCR buffer	4.0 μ L \times g, a, or c
dNTP mix (FDD)	0.3 μ L \times g, a, or c
H-T ₁₁ M (2 μ M)	4.0 μ L \times g, a, or c
Taq DNA polymerase	0.4 μ L \times g, a, or c
<hr/>	
32 μ L \times g, a, or c (total volume)	

- d. As an example, if there were 20 bands chosen for reamplification from FH-T₁₁G, g would be 22 (20 \times 10% = 22). A core mix should be made for 22 bands by multiplying the numbers from **step c** by 22:

Distilled water	23.3 μ L \times 22 = 512.6 μ L
10X PCR buffer	4.0 μ L \times 22 = 88 μ L
dNTP mix (FDD)	0.3 μ L \times 22 = 6.6 μ L
H-T ₁₁ M (2 μ M)	4.0 μ L \times 22 = 88 μ L
Taq DNA polymerase	0.4 μ L \times 22 = 8.8 μ L
<hr/>	
32 μ L \times 22 = 704 total volume	

- e. Make appropriate amounts of core mixes for both FH-T₁₁A and FH-T₁₁C.
14. After core mixes are made, aliquot 32 μ L into 0.2-mL tubes (individually, as strip tubes, or in a 96-well plate) labeled with the proper band name.
15. Add 4 μ L of the corresponding cDNA template from **step 11** as well as 4 μ L of the corresponding H-AP primer.
16. Place the reamplification reactions on the thermal cycler and perform using same conditions as FDD-PCR.
17. Make a 1.5% agarose gel with ethidium bromide by adding 1.5 g of agarose to 100 mL of 1X TAE. When the agarose/1X TAE mix cools to approx 50°C (barely

touchable by hand), add 3 μL of ethidium bromide (EtBr), swirl to mix, and pour solution into plastic agarose-casting tray.

18. Add 30 μL of the reamplification reaction to 5 μL of agarose DNA loading dye in a 0.5 mL microfuge tube. Load the 35 μL volume onto the 1.5% agarose gel. Save the remaining 10 μL of the PCR samples at -20°C for future cloning.
19. Electrophorese at 70 V for approx 45–60 min.
20. Confirm correct cDNA reamplification by visualizing gel using a UV transilluminator. Reamplified band should be approx the same size as the band cut from the original FDD gel.

After successful reamplification, each band must be confirmed to be a “real” difference by Northern blot or another technique. In addition, the band will need to be sequenced to determine if it is a known or a novel sequence. The order in which these are done can vary and is generally up to the preference of the researcher. Direct sequencing of the reamplified PCR products can sometimes be done here (*see Note 25*), but a cloning step is recommended first. The following steps are presented in the recommended order, but this can be modified based on the situation.

3.7. Cloning of Reamplified PCR Products

Clone differentially expressed cDNAs into recommended PCR-TRAP cloning vector, or other suitable cloning vector, following manufacturer’s protocol. The PCR-TRAP cloning vector (*see Note 26*) protocol is listed next for simplicity.

3.7.1. Ligation

The reamplified PCR products from differential display should be used directly for cloning without any post-PCR purification, modification, or dilution. Gel-purified PCR products contain inhibitors of DNA ligase and will lead to significantly reduced cloning efficiency (*see Note 27*).

1. For a 20 μL ligation reaction, add in order:

dH ₂ O	10 μL
10X ligase buffer	2 μL
PCR-TRAP Vector	2 μL
PCR product	5 μL
T4 DNA ligase (add last)	1 μL
<hr/>	
20 μL	

2. Mix well by finger tipping. Spin briefly.
3. Ligate overnight at 16°C .
4. Use directly for transformation or store at -20°C .

3.7.2. Transformation

1. Thaw the GH-competent cells in ice-water slush for 10–15 min. While the cells are melting, label the appropriate number of 1.5-mL microfuge tubes and set on ice. Quickly mix the cells by finger-tipping and aliquot 100 μ L into each 1.5 mL microfuge tube. Immediately refreeze (at -70°C) the remaining competent cells for future use.
2. Spin ligation tubes briefly to collect condensation. Add 10 μ L of each ligation mix to a tube containing the competent cells.
3. Mix well by finger-tipping and incubate on ice for 45 min.
4. Heat-shock the cells for 2 min at 42°C and then set the tubes back on ice for 2 min.
5. Add 0.4 mL of LB medium without tetracycline and incubate the cells at 37°C for 1 h. It is important that no tetracycline be in the LB during this step because the bacteria with recombinant plasmids need time to express the tetracycline-resistance gene (*see* **Note 28**). It is recommended that the LB-Tet plates be warmed up at 37°C for 1 h before plating, so this is a good time to do so.
6. After vortexing briefly, plate 200 μ L of cells on an LB-Tet plate (containing 20 $\mu\text{g/mL}$ of tetracycline). Store un plated cells at 4°C if re-plating is needed within 1 wk.
7. Once the plate surface is dry, incubate the plate upside down overnight at 37°C .
8. Score the Tet^R colonies and save the plate upside down at 4°C for further analysis.

3.7.3. Checking for the Insert

Checking for plasmids containing a DNA insert is easily done by the colony-PCR method using primers that flank the cloning site of the PCR-TRAP Vector. Therefore, it is unnecessary to wait another day before plasmid miniprep and restriction enzyme digestion can be performed.

3.7.3.1. COLONY LYSIS

1. On the bottom of the plate, number each Tet^R colony to be analyzed and then aliquot 50 μ L of colony lysis buffer into a corresponding microfuge tube.
2. Pick each colony with a clean pipet tip (try not to pick too much of the colony; a tiny amount that can be seen by the eye is usually more than enough) and transfer the cells into the colony lysis buffer of the corresponding numbered tube.
3. Incubate the tubes in boiling H_2O for 10 min.
4. Spin at room temperature for 2 min to pellet the cell debris, then transfer the supernatant into a clean tube with corresponding number.
5. Use the lysate immediately for PCR analysis or store at -20°C for future amplification.

3.7.3.2. PCR REACTION

1. For each colony lysate add (*see* **Note 29**):

dH ₂ O	20.4 μ L
10X PCR buffer	4.0 μ L
dNTPs (250 μ M)	3.2 μ L
Lgh primer	4.0 μ L
Rgh primer	4.0 μ L
Colony lysate	4.0 μ L
<i>Taq</i> DNA polymerase	0.4 μ L
	<hr/> 40 μ L

Mix well and add 30 mL mineral oil if required by the thermal cycler.

2. PCR parameters are as follows: 94°C for 30 s, 52°C for 40 s, 72°C for 1 min for 30 cycles, followed by a 5 min extension at 72°C, and a final incubation at 4°C. For checking cloned PCR products longer than 700 bp, increase the elongation time at 72°C from 1 to 2 min.
3. Analyze 20 μ L of the PCR product on a 1.5% agarose gel with EtBr staining, while saving the rest for sequencing. Plasmids containing an insert should result in an easily visible band. Verify the insert size by comparing the molecular weight of the PCR product before and after cloning. The PCR product after colony PCR should be 120 bp larger than the original PCR insert before cloning owing to the flanking vector sequence being amplified.
4. The bands should then be purified from the agarose gel using a QIAEX II kit and saved for Northern blot probe generation using GenHunter's HotPrime® DNA Labeling Kit.

3.7.4. Storing the Cloned PCR Products

1. After a plasmid has been determined to contain an insert of interest, the corresponding Tet^R colony should be re-streaked to single colonies on a new LB-Tet plate:
 - a. Locate the colony marked with the number on the original plate, pick it with a clean pipet tip, and streak the cells on a new LB-Tet plate.
 - b. Change to another clean tip, rotate the plate 90°, and streak a second time in order to obtain single colonies.
 - c. Incubate the plate overnight at 37°C.
2. Inoculate a single Tet^R colony into a 5 mL LB culture (no Tetracycline, *see* **Note 29**) and use 3 mL for plasmid miniprep. Save the remainder for glycerol (50%) cell stock at -70°C.

3.8. Sequencing of Cloned PCR Products

If using the PCR-TRAP Cloning System, sequencing can be performed utilizing vector-specific primers such as Lseq/Rseq or Lgh/Rgh. If using a clon-

ing vector other than the one recommended, consult manufacturer's guidelines for sequencing instructions.

1. For bands that are the correct size, purify the remaining 20 μ L of the saved colony PCR reaction using the QIAquick PCR Purification Kit.
2. This purified product can then be used directly for sequencing.
3. Determine the sequence of your band by finding the H-AP and H-T₁₁M sequences. The band can be cloned in either direction into the PCR-TRAP vector, so ensure that the sequence is in the correct orientation.

3.9. Confirmation of Differential Gene Expression by Northern Blot

To confirm differential expression of the selected cDNAs, Northern blot analysis is suggested rather than other confirmation techniques such as reverse northern hybridization (19), quantitative RT-PCR, or real-time PCR. The Northern blot technique is technically simple and straightforward in approach, requiring no manipulation of the RNA sequences from which differential gene expression has been detected. Additionally, Northern blot analysis is the most accepted confirmation technique for differential gene expression, often being referred to as the "gold standard" of gene expression confirmation assays. If using the recommended PCR-TRAP cloning vector, the probe template is produced by a PCR reaction of the cDNA construct within the cloning vector. The required primers are supplied with the cloning system. Additionally, the HotPrime DNA Labeling Kit, a random prime labeling kit with major improvements over the traditional random priming kit, is suggested. It is specifically designed to efficiently label DNA probes isolated from DD for Northern blot analysis. This method makes use of random decamers, rather than the traditional hexamers used in random priming, incorporates the anchored oligo-dT primers (H-T₁₁M) into the labeling buffer to ensure full-length anti-sense cDNA probe labeling, and uses radioactive dATP to take advantage of the AT-rich nature of DD bands. These improvements greatly increase the chance for signal detection on the Northern blot analysis.

3.9.1. Generation of cDNA Probes

The product that was gel-purified with the QIAEX II kit in **Subheading 3.7.3.2., step 4** will be used as a template to generate a probe using the HotPrime DNA Labeling Kit. If a different vector was used for cloning, consult manufacturer's suggestions for generating cDNA probes for Northern blot hybridization.

3.9.2. Labeling of cDNA Probes

1. If using the recommended HotPrime DNA Labeling Kit, thaw all components completely and immediately set them on ice.

2. Set up the following reaction in a 1.5-mL microfuge tube with a locking cap (so the cap will not loosen during boiling):

Distilled water	11 μL
10X Labeling buffer	3 μL
DNA template to be labeled (10–50 ng)	7 μL

3. Incubate the mixture in a boiling water bath for 10 min.
4. Quickly chill the tubes on ice. Spin the tube briefly to collect the condensation.
5. To the reaction, add the following *in order*:

dNTP (-dATP) (500 μM)	3 μL
α -[^{32}P] dATP (3000 Ci/mmol)	5 μL
Klenow DNA polymerase	1 μL

If using α -[^{32}P] dCTP instead of α -[^{32}P] dATP, substitute dNTP (-dCTP) for dNTP (-dATP).

6. Incubate for 20 min at room temperature, followed by incubation at 37°C for an additional 10 min.
7. Add 6 μL of the Stop buffer and mix well.
8. Purify the labeled probe with a Sephadex G50 column. Collect the purified probe in a 1.5-mL microfuge tube with a lock-on cap. Count 1 μL of labeled probe in a scintillation counter. A total of 10 million or more CPM can be obtained for most of the labeled DNA probes.

3.9.3. Probe Hybridization

1. Protocols for the preparation of a denaturing agarose (RNA) gel, including sample loading and electrophoresis conditions, and RNA transfer to nitrocellulose or nylon membrane has been previously described (20).
2. If the prehybridization buffer has been stored at -20°C, thaw at 37°C for 20 min.
3. Denature the salmon sperm DNA by incubating for 10 min in a boiling water bath.
4. Add salmon sperm DNA (to a final concentration of 100–200 $\mu\text{g/mL}$) in the prehybridization solution. Mix well.
5. Use 5 mL of prehybridization solution or enough to cover the membrane.
6. Prehybridize at 42°C for at least 4 h.
7. Denature the purified probe in a 1.5-mL microfuge tube with a lock-on cap (otherwise the cap may loosen) by boiling for 10 min in a water bath.
8. Chill on ice for 2 min.
9. Spin down the condensation and add the probe directly to the prehybridization solution.
10. Hybridize overnight.
11. Carefully decant the radioactive hybridization solution and dispose of in an appropriate container for radioactive waste.
12. Wash with 1X SSC containing 0.1% SDS *twice* at room temperature, each time disposing of wash solution in an appropriate container.

13. Wash for 15–20 min with 0.25X SSC containing 0.1% SDS *prewarmed* to the final washing temperature of 50–55°C.

3.9.4. Blot Exposure

1. Blot the membrane dry with paper towels and cover using UV-transparent plastic wrap.
2. Expose blot to single emulsion film with intensifying screen at –70°C for best signal detection.

It is hoped that most of the bands will be confirmed to show differential gene expression. Those that are confirmed are considered “real” differences as opposed to any “false positives” (*see Note 30*). The real differences, of course, will warrant further study and require downstream functional characterizations, where some of those mysteries of life can be pieced together.

4. Notes

1. Nonanchored oligo-dT primers have been used for DD, but their disadvantages far outweigh the advantage of needing only one primer for RT and PCR. Without the non-T base at the 3' end of the primer to “anchor” their position, they can anneal anywhere on the poly-A tail for PCR and will thus create many different sized DNA fragments for the identical cDNA species. This leads to a background smear, which is aesthetically unappealing, but more importantly will create problems for downstream reamplification of the wrong cDNA.
2. Although, poly-A+ RNA (mRNA) is what is actually being reverse transcribed in DD, it is rarely used as the RNA input. It can be purified and used for DD, but it provides no significant advantages and therefore total RNA is the preferred RNA source for DD for a number of reasons. First, it is much easier to purify than poly-A+ (mRNA), because simple RNA isolation reagents exist from many commercial sources, including RNeasy. Most of the protocols for purifying mRNA require purification of total RNA first, so it requires additional steps. Second, total RNA allows for easy evaluation of overall RNA integrity by running an “RNA gel” and visualizing the ribosomal RNA bands. If these bands are sharp and without a background smear, it can be assumed that the mRNA is also intact. There are ways to evaluate mRNA integrity, but they require expensive and sophisticated instruments such as the Agilent Bioanalyser. Finally, the methods used for mRNA purification generally require an oligo-dT binding step so that only the mRNA will be captured. This always leads to some oligo-dT contamination in the RNA sample, which will cause problems for the same reasons listed in **Note 1**. For all these reasons, total RNA is the RNA type of choice for DD.
3. The RNeasy reagent is a simple mono-phasic solution for rapid isolation of intact total RNA that is similar to other phenol/guanidine thiocyanate-based RNA isolation products, but has several major advantages. These include special cell lysis chemicals giving better yield, a yellow color allowing easier visualization during phase separation, and better stability with less corrosiveness. The high-quality RNA isolated can be used for DD, Northern blot and reverse Northern blot analysis, and other applications.

4. During RNA isolation from cells, it is crucial to completely remove any residual PBS after rinsing. Otherwise, the ratio of RNAPure to cells will be altered. Let the plate sit on an angle for 1 min and remove the residual PBS with a 1000- μ L pipet.
5. During RNA purification steps, many of the centrifugation steps are done at 4°C. We put our centrifuge in the refrigerator a few hours before these steps will be done. However, we have noticed that if you leave the centrifuge in the refrigerator continuously, it will not spin as fast. We assume this would be caused by either temperature or moisture. Therefore, if you are using a standard lab centrifuge designed for room temperature use, do not keep the centrifuge in the refrigerator long-term.
6. When removing the upper phase, it is crucial that you do not touch the interphase, which may contain proteins including RNases/DNases. It is much better to lose some RNA, but ensure that what RNA you do retrieve will be free of RNase/DNase, than to try to get as much of the upper phase as possible and risk RNase/DNase contamination.
7. Because tissues generally contain higher amounts of RNases than cells, we have noticed that a second-extraction phenol extraction step will significantly improve the DD results in terms of reproducibility and overall quality. This second extraction can be done directly after taking the upper phase and using more RNAPure reagent. Just add 1 mL of RNAPure reagent per 100 μ L of upper phase and follow the protocol starting at **Subheading 3.1.2.** again.
8. For the DNase I digestion step at 37°C, we recommend sticking to this 30-min time as closely as possible in case there is any RNase contamination. However, it is also crucial to do the full 30-min incubation to completely digest all DNA.
9. We have found that Phenol/ CHCl_3 (3:1) is superior to Phenol/ CHCl_3 (1:1) or Phenol/ CHCl_3 /isoamyl alcohol (25:24:1). However, these other options can be used, but the extraction should be repeated twice to insure complete removal of proteins. Phenol/ CHCl_3 /isoamyl alcohol is normally used for DNA or plasmid purification. It is recommended that all reagents for RNA work be separated from DNA work to avoid RNase contamination.
10. There are nonphenol/chloroform-based based protocols to inactivate or remove DNase including heat-inactivation, chemical-inactivation, or column-based purification. However, phenol/chloroform-based purification is the gold standard for protein removal and the only way to ensure that all DNase is removed. The other protocols may inactivate or remove most of the DNase, but for RT-PCR applications, even minute amounts of DNase will cause major problems with results. Therefore, we only recommend phenol/chloroform-based purification.
11. To check for RNA integrity, look for the clear appearance of the ribosomal RNA bands, with little to no smearing. RNA from different species can look significantly different, but mammalian RNA should have 28S and 18S rRNA bands in close proximity at the top of the gel and a 5S rRNA band lower. If the RNA appears degraded, this can be caused by many things:

- a. RNA was degraded before treatment with DNase I. Check integrity at all stages (before digestion, after digestion, after Phenol/CHCl₃ extraction, and so on). Make sure RNA is stored at -80°C at concentrations of at least $1\text{ }\mu\text{g}/\mu\text{L}$.
 - b. DNase I was contaminated. DNase I from many vendors contain detectable RNase contamination. The DNase I from the MessageClean Kit is guaranteed to be RNase-free.
 - c. RNA was degraded by reagents or equipment. Make sure all solutions and buffers are made with DEPC-treated dH₂O and all vessels, including tubes, tips, and gel boxes, are free of RNase.
 - d. The RNA sample itself is contaminated with RNase. This is a common problem with RNA extracted from large amounts of tissue, which is why at least two extraction steps are recommended for tissues. To confirm RNase contamination, incubate RNA with $1\text{--}2\text{ mM MgCl}_2$ in Tris-Cl, pH 8.0, at 37°C for 30 min. This will activate any RNase in the RNA. If this is confirmed, if enough “uncleaned” RNA remains, do an additional phenol/CHCl₃ extraction with RNA sample following the same procedures in **Subheading 3.2.2**. If not, start a new RNA extraction and increase the RNA extraction solution (RNApure) to tissue ratio and do an additional phenol/CHCl₃ extraction step.
 - e. The RNA sample sometimes appears to be degraded after agarose gel analysis, when the actual problem is the pH of the buffer, too much salt in the RNA, or bad loading dye, which has caused the ribosomal RNAs (28S and 18S) to migrate strangely. We recommend using the RNA Loading Mix. Confirm the pH of the MOPS buffer, which should be between pH 6.5 and 7.0. Also, make sure formaldehyde is added to the gel and the RNA sample is denatured by incubating in RNA Loading Mix at 65°C for 10 min before loading.
12. RNA samples should be freshly diluted with dH₂O or DEPC-treated H₂O to $0.1\text{ }\mu\text{g}/\mu\text{L}$ directly before RT reaction setup. Do not reuse the diluted RNA after freezing and thawing because the RNA will be degraded and yield poor results.
 13. For the RT reaction, the initial 65°C incubation is intended to denature the RNA secondary structure. The final incubation at 75°C is to inactivate the reverse transcriptase without denaturing the cDNA/mRNA duplexes. Therefore “hot start” PCR is neither necessary, nor helpful for the subsequent PCR reactions using cDNAs as templates.
 14. If not using the recommended thermal cycler, you may need to adjust the denaturation (94°C) time to 30 s.
 15. The PCR setup for H-AP primers 73–80 can be done at the same time for all 3 FH-T₁₁M primers so they can all be put on one 96-well plate.
 16. Gel debris and streaks on the glass plates will usually fluoresce and can cause major background problems. Therefore, thorough cleaning is required.
 17. If overnight gel polymerize is done, plastic wrap, such as Saran Wrap, should be used to prevent the gel from drying out.
 18. During sample gel-loading, it is crucial that the urea in the wells be completely flushed immediately before loading your samples. Because urea is heavier than water, it will fall to the bottom of the well fairly quickly. If a sample is loaded

without flushing a well, it will sit on top of the urea, which in turns causes strange migration and poor resolution. For best resolution, flush every 4–6 wells loaded using a syringe or pipet while trying not to disturb samples that have already been loaded.

19. Fluorescent dyes are light-sensitive. We recommend keeping primers and samples in the dark or covered with aluminum foil. While running the gel, the apparatus should also be kept in the dark as much as possible. This can be done by running gels in a dark room or using a cardboard box to cover the entire apparatus.
20. When scanning the gel, it is best to remove the gel tape, spacers, and comb, which will fluoresce and can cause background problems. However, if you think you might run the gel longer for better separation, you should do a quick scan before removing gel tape, spacers, and comb to determine if it has been run long enough.
21. To separate the glass plates, we have found that small plastic wedges, which can be purchased from several gel companies, work well. It is important to do this slowly to make sure the gel is sticking only to one side.
22. When printing out the real-size image, you will need a paper large enough to fit the whole gel. We use 11×17 paper on an ink-jet printer, which allows plenty of space for the entire gel. If necessary, you could also print the gel on two to three pieces of paper and tape them together.
23. When selecting bands to cut, if there is a chance that pursuit of the band is worthwhile, it is recommended to cut it. Later, a decision can be made whether or not to reamplify that band. However, if one later decides to pursue a band that was not cut, the gel will have to be run again because gels can only be stored for a few days before drying out. When a large quantity of gels is being run, it usually makes sense to run all the gels first, cutting any interesting bands along the way, and storing those bands in the refrigerator. When all gels have been completed, a decision can be made on which bands are worthwhile reamplifying and then they can be done together.
24. For the reamplification reaction, note that the unlabeled (without 5' fluorophore) H-T₁₁M primers are used. Otherwise, the fluorophore can interfere with future cloning.
25. Direct sequencing can sometimes be done following successful reamplification. If the reamplified product is a single, clean band, direct sequencing with the H-AP primer can work, generally about 50% of the time. However, if the reamplified product has multiple bands, a cloning step will have to be done first.
26. The PCR-TRAP Cloning System is by far the most efficient cloning method for PCR products that we have tested. It utilizes a third-generation cloning vector that features positive selection for DNA inserts. Only recombinant plasmids confer antibiotic resistance. The principle of this unique cloning system is based on the phage Lambda repressor gene, cI, which is cloned on the PCR-TRAP vector and codes for a repressor protein. The repressor protein binds to the Lambda right operators Or1 to Or3 of the *cro* gene, thereby turning off the promoter that drives the Tet^R gene on the plasmid. Therefore, cloning of the PCR products

directly, without any post-PCR purification, into the cI gene leads to inactivation of the repressor gene, thus turning on the Tet^R gene. This allows the *Escherichia coli* containing recombinant plasmids to grow on Tet plates.

27. For PCR product cloning of DD fragments, we do not recommend any post-PCR purification, modification, or dilution. However, if you decide to gel-purify, cloning can still be done, but with much-reduced efficiency. GenHunter always recommends using the PCR product directly in the ligation step. Some reagents used in gel purification, such as NaI, are potent inhibitors of DNA ligase. If purified PCR products must be used (whether by gel or column purification), adding more ligase (two to five times as much) may help.
28. Although PCR-TRAP is a high copy-number cloning vector, cells harboring the plasmid are more sensitive to tetracycline in liquid culture than on plates. Do not add any tetracycline in liquid cell culture, because it may significantly inhibit cell growth.
29. For the colony-PCR reactions, it is recommended that a core mix containing everything except the colony lysate be made in order to minimize pipetting errors and to be able to analyze many colony lysates at one time.
30. If a band chosen from DD does not show differential expression on a Northern blot, it does not mean that it is necessarily a "false positive." We have seen several examples where bands show no noticeable differential expression on Northern blots, but upon review, something else is involved such as polymorphism at primer binding site, short sequence deletion/insertion, splicing difference, and so on. The message is if a band looks convincing on the DD gel, but does not show differential expression by Northern blot, it could be a false positive, but could also be something very interesting, and worth pursuing.

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