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Screening differentially expressed cDNA clones obtained by differential display using amplified RNA

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ABSTRACT

The major obstacle of differential display is not the technique itself but rather the post-differential display issue of discriminating between false positives and the truly differentially expressed mRNAs. This process is arduous and requires large amounts of RNA. We present and validate a method which allows one to screen putative positives from differential display analysis using only micrograms of total RNA. More importantly, we demonstrate that cDNA probes generated from amplified RNA are representative of the starting mRNA population and can be used for differential screening of mRNA species at a detectable limit of sensitivity of ≥1/40 000.

Differential display (DD; <BBR ID=1>–<BBR ID=3>) is currently the method of choice among many investigators for identifying differentially expressed mRNAs because it identities mRNAs independent of prevalence, uses small amounts of RNA (<5 µg total RNA), identifies both increases and decreases of mRNA levels and has rapid output (<BBR ID=4>). As stated by Debouck (<BBR ID=5>), the downstream verification process for DD is the most time consuming and requires significant amounts of RNA (i.e., a great deal more RNA than actually needed to perform the initial differential display analysis).

In general, DD can generate many false positives and therefore without a high throughput method to screen for the truly differentially expressed cDNAs, the investigator can easily be overwhelmed. Screening methods such as Northern assay, RNase protection assay, quantitative PCR, differential screening (<BBR ID=6>) and *in situ* hybridization are usually not optimal for large scale screening operations because of either the large amounts of RNA required and/or the necessity to test each putative positive one at a time.

Here we present and validate an improved screening method that combines differential screening with the use of amplified RNA (aRNA) (<BBR ID=7>,<BBR ID=8>) generated from 5 µg total RNA. The use of radiolabeled amplified RNA (aRNA) has already been shown to enable the detection of differential expression of mRNAs (<BBR ID=7>,<BBR ID=8>). However, an examination to determine whether aRNA is representative of the original mRNA population has not been published. In addition, for use as a screening tool for putative positives from DD analysis, the original aRNA method has two disadvantages: (i) in our hands, RNA probes produce higher backgrounds than cDNA probes, and (ii) because the aRNA is labeled, it has a short half-life. As shown below, to avoid these problems we have generated cDNA probes from unlabeled aRNA that was obtained from total RNA and demonstrate that such cDNA probes are representative of the starting mRNA population [i.e. poly(A)], and can be used to detect mRNA expression with a limit of sensitivity of at least 1:40 000.

To validate this method, we chose 10 distinct cDNAs previously identified by differential display as either differentially expressed (clones l–5) or unchanged (clones 6–10) in HeLa cells in response to interferon-γ (IFN-γ) treatment (<BBR ID=4>). Each cDNA was spotted onto four replicas of nylon membrane at five concentrations ranging from 60 ng to 6 µg. Four cDNA-labeled probes were synthesized from either 2 µg poly(A) RNA extracted from untreated or IFN-γ-treated cells, or 2 µg aRNA (amplified from 5 µg total RNA) from untreated or IFN-γ-treated cells. Total RNA was prepared using RNAzol B solution (Tell-Test) and mRNA was prepared from total RNA using FastTrack 2.0 mRNA Isolation kit (InVitrogen).

RNA amplification was performed following a previously described protocol (<BBR ID=8>) with a few modifications. Double-stranded cDNA was made using 5 µg total RNA from untreated or IFN-γ- stimulated HeLa cells, and 94 ng of $T7(dT)_{15}$, (5′-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T_{15}-3′) using the Superscript Plasmid System for cDNA Synthesis (Gibco BRL). The cDNA was blunt-ended with T4 DNA polymerase; the reaction was stopped by incubation at 65°C for 10 min. The cDNA was then precipitated by ethanol precipitation in the presence of ammonium acetate and resuspended in H_2O. RNA amplification was performed with the whole cDNA reaction using the Ampliscribe T7 Transcription kit (Epicentre). Incubation was carried out for 4 h at 37°C and the aRNA was precipitated with ammonium acetate as follows: 0.5 vol 7.5 M ammonium acetate was added (no ethanol is added) to the reaction, the mixture was chilled on ice for 15 min, and centrifuged at 14 000 r.p.m. for 30 min at room temperature, and the subsequent aRNA pellet was resuspended in H_2O (using this precipitation method effectively eliminates free nucleotides). ^{32}P-labeled cDNA probes were made with 2 µg poly(A) RNA or aRNA using Superscript Preamplification System (MMLV-reverse transcriptase; Gibco BRL). The primer consisted of 1 µg oligo (dT)12-18 (Gibco BRL) when poly(A) RNA was used and 740 ng random hexamers pd(N)6 (Pharmacia) when aRNA was used. The reaction was performed in 25 µl final volume using 2.5 µl 10× PCR buffer, 2.5 µl 25 mM $MgCl_2$, 2.5 µl 0.1 M DTT, 1 µl dGTP/dATP/dTTP mix (20 mM of each), 1 µl dCTP (120 µM)

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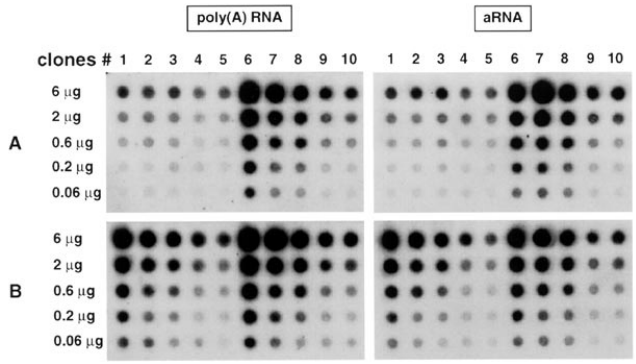

Figure <FIGR ID=1>. Differential screening of selected cDNAs using ^{32}P-labeled cDNA probes synthesized from poly(A) RNA or aRNA from untreated cells (**A**) and γ-IFN-stimulated cells (**B**). HeLa cells were stimulated twice, at day 0 and day 1, with 2.5 U/ml γ-IFN (Boehringer Mannheim) and RNA was extracted at day 2.

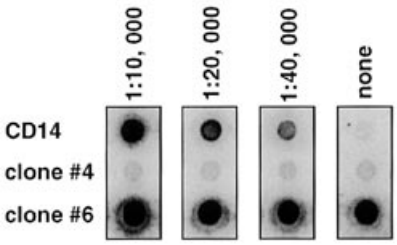

Figure <FIGR ID=2>. Sensitivity of differential screening performed with a ^{32}P-labeled cDNA probe generated from aRNA. CD14 cRNA was added to untreated HeLa cells aRNA at molar ratios of 1/10 000, 1/20 000 and 1/40 000. Clones #4 and #6 are negative and positive controls respectively (Fig. 1).

and 7.5 µl [α-^{32}P]dCTP nucleotides (3000 Ci/mmol, 0.5 mCi/reaction, Amersham), according to the manufacturer's instructions. RNA was then hydrolyzed by a 30 min incubation at 65°C in the presence of 3 µl 3N NaOH. The mixture was neutralized by adding 10 µl 1 M Tris–HCl (pH 7.4), 3 µl 2N HCl and 9 µl H_2O. Unincorporated nucleotides were removed by passage through a G50 Sephadex column (Boehringer Mannheim).

Plasmid DNAs (Fig. <FIGR ID=1>) or cDNA inserts amplified by PCR (Fig. <FIGR ID=2>) from clones 1–10 were transfered onto a nylon membrane (Maximum Strength Nytran, 0.45 mm; Schleicher & Schuell) using a 96-well Dot Filtration Manifold System (Gibco BRL). DNA was then denatured once for 5 min at room temperature (1.5 M NaCl, 0.5 M NaOH), neutralized twice for 5 min at room temperature (1.5 M NaCl, 1 M Tris–HCl pH 7.4), rinsed in 2× SSC and finally UV cross-linked once in a UV-stratalinker 2400 (Stratagene). Membranes were pre-hybridized in 1× Southern pre-hybridization buffer (5 Prime to 3 Prime, Inc.) containing 50% formamide and 100 µg/ml sheared salmon sperm DNA overnight at 42°C. Hybridization was performed in 1× Southern hybridization buffer containing 50% formamide and 100 µg/ml sheared salmon sperm DNA (5 Prime to 3 Prime, Inc.) overnight at 42°C in the presence of 10^7 c.p.m./ml cDNA probe. Blots were placed onto a phosphor screen for 1 day and results were analyzed using a PhosphorImager 445SI (Molecular Dynamics).

As shown in Figure <FIGR ID=1>, similar results are observed with cDNA-labeled probes derived from either unamplified or amplified RNA. Quantitative analysis of the hybridization via the PhosphorImager 445SI (data not shown) confirmed this observation: the relative signal intensities obtained with poly(A) RNA-derived probes were similar to the one obtained with aRNA-derived probes. However the overall specific activity of the aRNA-derived probes was, to some extent, lower than the one of poly(A)-derived probes, which is probably due to the fact that aRNA has a higher level of ribosomal RNA contamination than poly(A) RNA. In a subsequent experiment we have found similar results with 45 additional cDNAs from the HeLa +/– IFN-γ paradigm (data not shown). Yields of aRNA from 5 µg total RNA range from 10 to 50 µg. With 10 µg aRNA, five cDNA probes can be synthesized and 10 96-well format spotted membranes probed (two membranes back-to-back hybridization); thus at least 960 cDNAs can be differentially screened from 5 µg total RNA.

With regard to sensitivity, there is an obvious correlation between DNA concentration and sensitivity [similar results were found with amplified (PCR) inserts, data not shown]. In addition, the limit of detection for determining a differentially expressed mRNA appears to be between the prevalences of clone 3, which shows a 2.5-fold up-regulation and clone 4 which shows a 2.2-fold up-regulation, whereas the maximum difference observed for the negative controls is 1.3-fold (quantitative results obtained when 6 µg DNA is spotted onto nylon membrane, data not shown). Clones 1, 2 and 3 previously have been found to have a prevalence of 1/1500, 1/7800 and 1/54 000 respectively (<BBR ID=4>). To test the limit of detection directly, defined amounts of cRNA synthesized from human CD14 cDNA (not expressed in HeLa cells) were added to untreated HeLa cell aRNA prior to probe synthesis. As shown in Figure <FIGR ID=2>, the limit of the prevalence at which CD14 can be detected is at least 1/40 000 at which the detection signal is 7.5-fold the background signal.

In conclusion, we validate the use of aRNA in the differential screening method and demonstrate that this method can be used to detect mRNA with a frequency as low as l/ 40 000. This method will complement one of the main pitfalls of differential display and allow a wider use of the technique.

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REFERENCES

<BBR ID=1>
Liang,P. and Pardee,A.B. (1992) *Science*, **257**, 967–971.
<BBR ID=2>
Welsh,J., Chada,K., Dalal,S.A., Cheng,R., Ralph,D. and McClelland,M. (1992) *Nucleic Acids Res.*, **20**, 4965–4970.
<BBR ID=3>
Wang,X., Ruffolo,R.R.J. and Feuerstein,G.Z. (1996) *Trends Pharmacol.*, **17**, 276–279.
<BBR ID=4>
Wan,J.S., Sharp,S., Poirier,G.M.-C., Wagaman,P., Chambers,J., Pyati,J., Hom,Y., Galindo,J., Huvar,A., Peterson,P.A., *et al.* (1996) *Nature Biotech.*, **14**, 1685–1691.
<BBR ID=5>
Debouck,C. (1995) *Curr. Opin. Biotech.* **6**, 597–599.

<BBR ID=6>
Zhang,H., Zhang,R. and Liang,P. (1996) *Nucleic Acids Res.*, **24**, 2454–2455.
<BBR ID=7>
Eberwine,J., Spencer,C., Miyashiro,K., Mackler,S. and Finnell,R. (1992) *Methods Enzymol.* **216**, 80–101.
<BBR ID=8>
Van Gelder,R.N., Von Zastrow,M.E., Yool,A., Dement,W.C., Barchas,J.D. and Eberwine,J.H. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1663–1667.