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Global amplification of sense RNA: a novel method to replicate and archive mRNA for gene expression analysis

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Abstract

We have developed a procedure to amplify mRNA into sense RNA (sRNA) so as to create a regenerating biorepository representing the complex mRNA profile in the original sample. The procedure exploits the template-switching activity of reverse transcriptase to incorporate RNA polymerase binding sites upstream of single-stranded cDNA (ss cDNA). Limited PCR was used for double-stranded DNA (dsDNA) synthesis. sRNA was synthesized from PCR products by in vitro transcription (IVT). sRNA was evaluated by real-time reverse transcription (RT)-PCR. sRNA synthesis was successful with RNA from human cell lines and tissues, yielding 2000- to 2500-fold amplification of glyceraldeyde-3 phosphate dehydrogenase (G3PDH). The size of sRNA ranged from 3.0 to 0.1 kb. sRNA synthesis preserved the relative differences in plant mRNAs spiked at abundance ranging over 5 orders of magnitude (0.00001–0.1%). This reflects the high fidelity of sRNA synthesis for mRNA as low as 0.3 copies/cell. sRNA is amplified synthetic mRNA in the 5′→3′ direction; the appropriate template for any gene expression analysis.

Keywords: Gene expression profiling; RNA amplification; Antisense RNA; Sense RNA; Biomarkers

High throughput gene expression profiling methods have allowed biologists to take a broad and open approach to the discovery of novel markers for the early detection and classification of diseases. The same molecular markers and biochemical pathways may also result in novel targets for therapeutic or preventive drug design. Each ‘discovered’ disease marker requires careful validation in well-designed epidemiological studies to test the strength of the association or predictive value. Thus much of the work of translation research is involved with approaches to optimize both the population studied and the biological sample collected. Biorepositories linked to epidemiological data are crucial resources for the validation and discovery of markers. However, the finite amount of mRNA available from each sample and the lability of RNA during long-term storage substantially limit the numbers of studies that these priceless collections can support.

Several effective amplification approaches have been developed that allow gene expression profiling even on the few cells available from microdissection [1–4]. However, these methods do not address the problem of long-term storage of samples for gene expression profiling. The lability of RNA and the ubiquitous presence of ribonucleases lead to the inevitable compromise of RNA quality even if it is highly purified and stored at temperatures lower than −70°C in the presence of RNase inhibitors. Earlier approaches to archiving have relied on making DNA copies of the RNA. Such cDNA libraries are more amenable to long-term storage because of their increased stability; however, using these libraries directly in gene expression profiling assays is very complicated [5,6].

Our goal was to develop a method that would make RNA in biorepositories very close to a stable renewable resource

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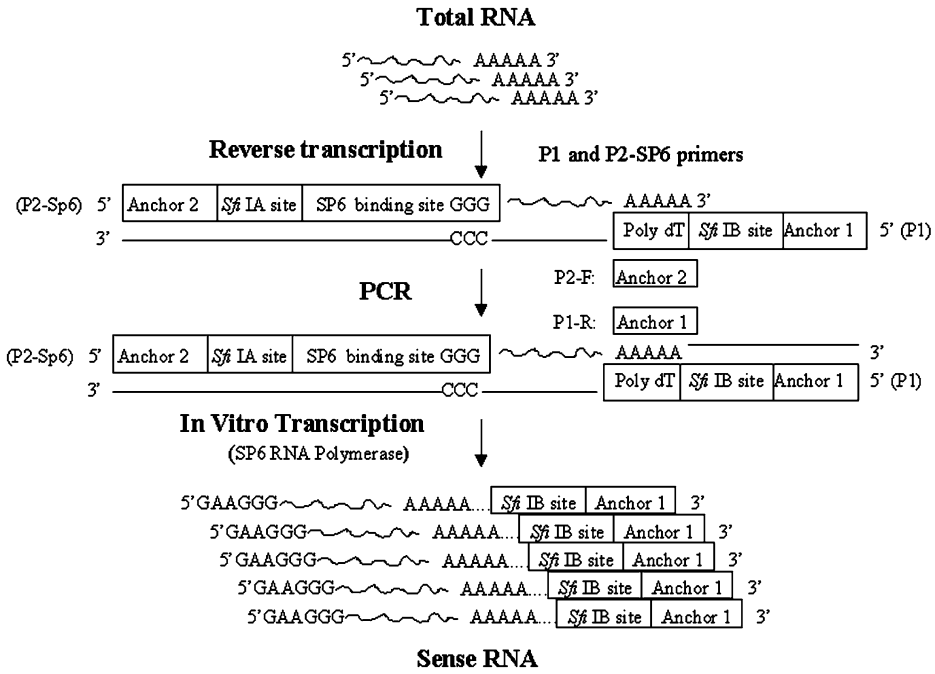

Fig. 1. Diagram of protocol for amplification of sense RNA (sRNA). Total RNA is reverse transcribed with P1 and P2-SP6 primers. SP6 RNA polymerase binding site is incorporated upstream of cDNA by the template-switching activity of reverse transcriptase enzyme. When the enzyme reaches the 5′ end of mRNA, it adds a 'CCC' triplet to the growing end of the cDNA. The added 'CCC' allows the P2-SP6 primer to bind, and the enzyme 'switches' to copy the DNA template provided by the P2-SP6 primer. Ds cDNA is generated by PCR using primers corresponding to sequences in the P1 and P2-SP6 primers. sRNA, which has the same directionality as the original mRNA population, is synthesized by IVT reaction using the PCR products.

that could be directly used in all approaches to gene expression profiling (e.g., differential display PCR, RT-PCR, RNase protection assays, microarrays). Key features include: (1) a modified poly(dT) primer to direct reverse transcriptase to poly(A) transcripts as well as to incorporate anchoring and restriction enzyme (cloning) sites; (2) use of the template-switching activity of reverse transcriptase to incorporate a second primer with anchoring, restriction enzyme (cloning), and RNA polymerase binding sequences at the 3′ end of the single-stranded first cDNA product; and (3) use of anchor-primed limited PCR to globally copy all products to ds cDNA products for archiving. When needed, the archived material is used as template for IVT from the upstream RNA polymerase site to yield amplified RNA in the sense direction (sRNA). The sRNA is amplified synthetic mRNA; the appropriate template for any gene expression profiling assay.

Results

As illustrated in Figure 1, the template-switching activity of the reverse transcriptase enzyme is used to incorporate an RNA polymerase binding site into the 3′ end of the ss cDNA product. This RT product is converted to ds cDNA using a limited-cycle PCR. We varied the number of PCR cycles from 3 to 18 on separate aliquots of the same total RNA source (Caski) to determine the optimal conditions for IVT. Amplification was determined from the crossing-point/threshold (C_p) values for real-time RT-PCR of G3PDH (an endogenous target) and reproducibility from the coefficient of variation (CV) of the C_p.

As shown in Table 1, the ds cDNA yield increased nearly 10-fold between 12 and 15 PCR cycles, and was further increased with 18 cycles. As expected, the CV was lowest for unamplified original total RNA (direct RT). For amplified synthetic sRNA (sRNA-RT), the CV decreased and amplification increased with increasing PCR cycles. Using these results, we selected 18 cycles of PCR as the standard

Table 1
Impact of PCR cycles on sRNA synthesis[a]

	PCR Cycle number					
	0[b]	3	9	12	15	18
Yield ds cDNA (ng)[c]	—	70	120	150	1120	5000
CV for G3PDH C_p (%)[d]	0.8	8.4	5.2	4.8	1.3	2.8
sRNA amplification[e]	ref	1×	24×	300×	1120×	2500×

[a] Aliquots of same Caski total RNA used in all experiments.
[b] Direct RT of total RNA, no PCR or IVT.
[c] Yield based on fluorometry.
[d] Coefficient of variation for real-time RT-PCR G3PDH C_p values (n = 3).
[e] The amplification of sRNA relative to direct RT was based on C_p values for RT-PCR for G3PDH and dilution factors (proportion of the PCR product used in the IVT reaction).

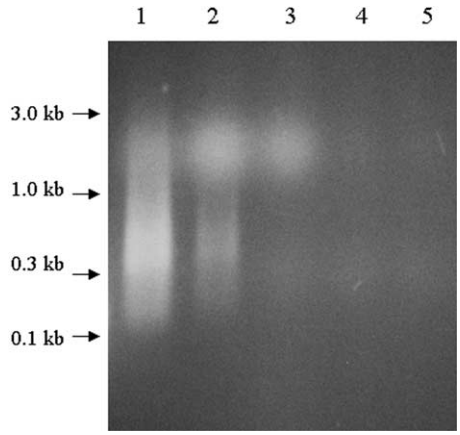

Fig. 2. Size distribution of sRNA products from Caski RNA. Denaturing agarose gel electrophoresis of 1 μl sRNA from IVT stained with ethidium bromide [17]. IVT reactions (lanes 1–5) were done with various templates. Lane 1, 100 ng PCR product; lane 2, 20 ng PCR product; lane 3, PCR product from no RT control (without reverse transcriptase); lane 4, PCR H_2O control; lane 5, no PCR template H_2O control.

conditions for preparing the ds cDNA intermediate for sRNA synthesis.

The size of the sRNA resulting from this protocol ranged from 3.0 to 0.1 kb, with intense smearing in the range of 1.0 to 0.3 kb (Fig. 2, lanes 1 and 2). The intensity of sRNA smear varied with the amount of ds cDNA in the IVT reaction. IVT reactions were successful when the amount of ds cDNA was increased up to 500 ng per reaction; however, inhibition was encountered when the entire RT product was amplified and used in the IVT reaction (data not shown). No products were visible when sRNA synthesis was done without reverse transcriptase in the direct RT or when PCR and IVT were done without templates (Fig. 2, lanes 3–5).

Figure 3 illustrates the results of real-time RT-PCR for G3PDH. Only reactions with templates from direct RT or sRNA-RT amplified. The C_p reflected the input amount of ds cDNA in the IVT (Fig. 3A, reactions 2 and 3). These products had the melting peaks and the expected size (450 bp) for the G3PDH transcript (Fig. 3B). The same protocol was used to synthesize sRNA from total RNA of several tissues (human kidney, cervical tumor, and placenta). Results were comparable to those obtained for Caski total RNA (data not shown).

Caski total RNA was spiked with varying dilutions of plant mRNAs (Table 2) to model mRNA abundance varying from 0.1 to 0.00001% of total mRNA, and sRNA was prepared from each sample. The C_p for each exogenous transcript was determined from real-time RT-PCR for the starting sample (direct RT) and the sRNA product (sRNA-RT). Figure 4A shows the regression lines for C_p against log (% total mRNA) for direct RT and sRNA-RT. The slopes, reflecting PCR efficiency, were nearly identical for amplified and unamplified products for all three transcripts. This indicates that sRNA maintains the relative differences in transcript abundance even for proportions as low as 0.00001% of total mRNA, the equivalent of 0.3 copies of mRNA per cell. Direct RT and sRNA-RT of all three plant

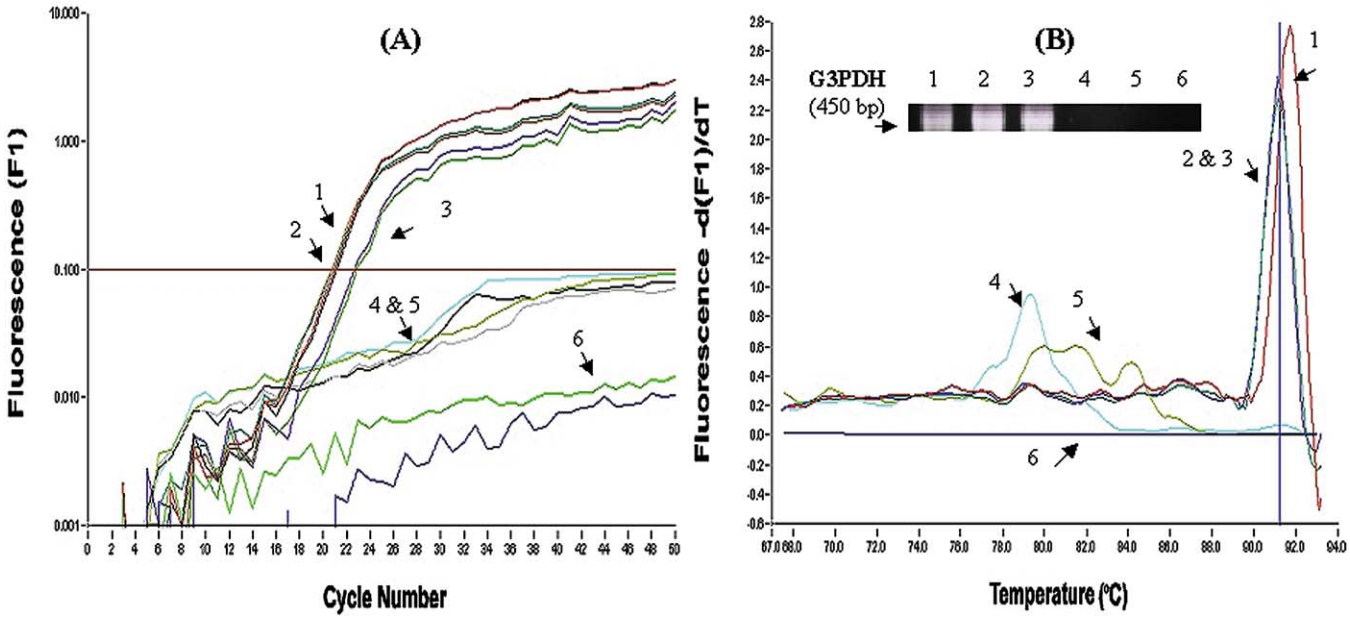

Fig. 3. Detection of G3PDH in sRNA by real-time RT-PCR. (A) Amplification curves for duplicate reactions show that only those with direct RT or sRNA-RT amplified G3PDH. Signals from negative control reactions 4–6 were below the noise band (horizontal line). Reactions were as follows: 1, direct RT; 2, sRNA-RT using IVT with 100 ng ds cDNA; 3, sRNA-RT using IVT with 20 ng ds cDNA; 4, sRNA-RT using IVT with PCR H_2O control; 5, sRNA-RT as in reaction 2 but without reverse transcriptase; 6, LightCycler reaction without template (H_2O control). There are two amplification curves (duplicates) per reaction. (B) Melting curve and gel electrophoretic analyses of G3PDH. LightCycler product showed that only reactions that showed amplification had specific melting peaks and the expected product size (450 bp). Melting peaks 1–6 and gel lanes 1–6 correspond to the same reactions 1–6 described in panel A.

Table 2
Scheme for spiking plant mRNAs in 1 μg Caski total RNA

Plant mRNA	pg (% of total mRNA)[a]		
	Sample 1	Sample 2	Sample 3
ERS	20 (0.1)	2.0 (0.01)	0.2 (0.001)
CAB	2.0 (0.01)	0.2 (0.001)	0.02 (0.0001)
RCA	0.2 (0.001)	0.02 (0.0001)	0.002 (0.00001)[b]

ERS, peach ethylene receptor; CAB, chlorophyll a/b-binding protein; RCA, RUBISCO activase.

[a] Assuming 2% mRNA in total RNA, 20 pg of exogenous mRNA into 1 μg total RNA will make up 0.1% of mRNA in total RNA.

[b] Based on size of RCA (0.5 kb), 0.002 pg = 7.5×10^3 molecules. Based on 40 pg of total RNA/cell, 1 μg total RNA corresponds to 2.5×10^4 cells [4]. RCA in this sample corresponds to 0.3 copies of mRNA/cell.

transcripts gave specific melting peaks and expected product sizes (Fig. 4B). No melting peaks or specific products in the gel were observed with any of the negative controls.

Discussion

We demonstrated a novel procedure for creating a regenerating biorepository representing the complex mRNA profile in the original sample. This method of amplifying sRNA was successfully applied to total RNA from human cell lines and tissues. Earlier described methods for amplified RNA synthesis attach a T7 RNA polymerase binding site at the 5′ end of cDNA product through an oligo-dT primer, and result in antisense RNA (aRNA) [7–10]. By contrast, we generate amplified sRNA because the RNA polymerase

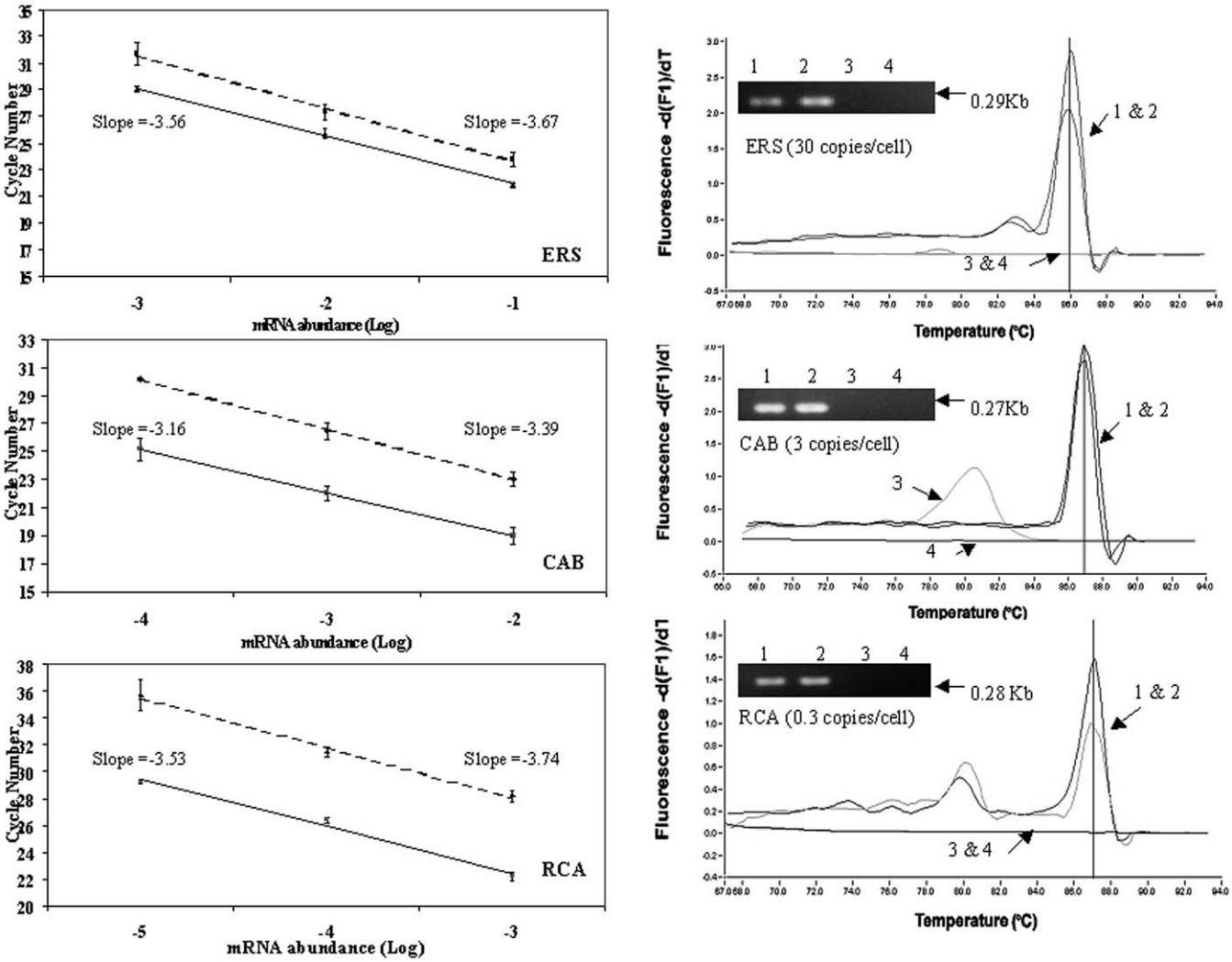

Fig. 4. Impact of sRNA synthesis on mRNA abundance. (A) Plant mRNAs were spiked to the same Caski total RNA to reflect abundance levels as shown in Table 2. The regression lines for ERS, CAB, and RCA transcripts indicate that sRNA maintains the relative differences in transcript abundance even for proportions as low as 0.00001% of total mRNA. Broken lines, direct RT. Solid lines, sRNA-RT. (B) Specific melting peaks and the expected product sizes (270–290 bp) verify that all levels, including the lowest levels, of all plant transcripts (0.3–30 copies/cell) were amplified. Negative controls did not show any melting peaks or product in the gel. Melting peaks for reactions: 1, direct RT; 2, sRNA-RT; 3, sRNA-RT without reverse transcriptase; 4, H_2O control reaction. Gel electrophoresis of LightCycler products: Lanes 1–4 correspond to the same reactions used for detection of specific melting peaks.

binding site is attached at the 3′ end of the cDNA using the template-switching activity of reverse transcriptase. Independent of the transcript sequence, when the enzyme reaches the 5′ end of mRNA it adds a 'CCC' triplet to the growing end of the cDNA [5]. The added 'CCC' allows the P2-SP6 primer to bind, and the enzyme 'switches' to copy the DNA template provided by the P2-SP6 primer. To optimize the template-switching activity, we used Moloney Murine Leukemia Virus (MMLV) reverse transcriptase deficient in RNase-H as described for SMART-PCR [5]. The sRNA produced is similar to aRNA in terms of the size distribution of products (3–0.1 kb) and the level of amplification (2000- to 2500-fold) [7,11].

The intermediary ds cDNA for aRNA synthesis is usually prepared using the multiple-enzyme reactions first described by Gubler and Hoffman [8,12]. This approach has the potential for bias because of the multiple enzymes, and underrepresentation of open reading frames was shown with cDNA library construction [5]. We generated ds cDNA using PCR with limited cycles to avoid this problem. In agreement with SMART-PCR and Poly(A) PCR [3,6,13,14], our results show the reproducibility of sRNA synthesis and that the relative differences in transcript levels between samples was maintained by this procedure. SMART-PCR-based approaches have also been recently used to generate ds cDNA for aRNA synthesis [9,10].

Most aRNA procedures also have problems with the synthesis of nonspecific products, attributed to the carryover of primers containing the RNA polymerase binding site to the IVT reaction [15]. We detected no nonspecific products in sRNA synthesis (Fig. 2, lane 3) by using 10-fold less P2-SP6 primer in the RT reaction, and a fraction of the RT and purified PCR products in the IVT reaction.

The spiking experiments demonstrate the high fidelity of sRNA synthesis. The plot of C_p values as a function of the abundance of mRNA spikes (Fig. 4A) shows that sRNA synthesis is linear with respect to mRNA abundance ranging over 5 orders of magnitude (0.00001–0.1% w/w) and will faithfully amplify extremely low-abundance transcripts (0.3–30 copies/cell) in the original samples. The specific detection limit of sRNA is similar to that reported for aRNA amplification (1–3 copies/cell) by hybridization to oligonucleotide arrays [16]. This level of sensitivity with sRNA should allow detection of the major class of low-abundance mRNAs (<5 copies/cell) in a sample [17].

We chose to use SP6 RNA polymerase in this protocol because the trinucleotide 'GGG' at the 3′ end of its highly conserved promoter structure [18] and the added 'CCC' residues at the 3′ end of the cDNA would hybridize and maximize template switching (Fig. 1). T7 or T3 RNA polymerases binding sites could be incorporated with appropriate design of the P2-SP6 and P2-F primers. We have tried sRNA synthesis with T3 RNA polymerase and found product size and level of amplification to be similar to those of SP6 RNA polymerase (data not shown).

There is little doubt that residual ribonucleases can unpredictably compromise the quality of RNA from clinical samples during long-term storage. Although converting RNA into cDNA may improve stability, conventional RT products are not renewable and lack features that permit global amplification and construction of cDNA libraries. The introduction of specific transcription and restriction enzyme sites into the intermediary ds cDNA in our protocol results in a multifunctional product that can be used to archive and amplify the original RNA. These PCR products can be renewed through reamplification with the same forward and reverse primers. Because the ds cDNA contains recognition sites of *Sfi*I restriction enzyme, they are also suitable for the construction of cell type-specific cDNA libraries as outlined by Zhu et al. [5].

Because the sRNA from IVT is in the same orientation as mRNA, we anticipate that it should be useful in all standard formats of expression profiling technologies such as northern blots, RNase protection assays, conventional differential screening, different microarray platforms, serial analysis of gene expression, and differential display PCR. Results presented here have shown that sRNA is an excellent template for real-time RT-PCR, but additional studies are required to evaluate the efficacy of sRNA in other gene expression assay formats. Similarly, although the basic strategy is broadly applicable to all samples, it is probable that those preserved or fixed in organic solvents, with low mRNA content or partial degradation, or with inhibitors, may require additional optimization to improve the efficiencies of the reactions.

Materials and methods

RNA preparation

We extracted total RNA from monolayers of human cervical cancer cell line Caski (American Type Culture Collection, Rockville, MD) using the modified guanidinium thiocyanate method [19] followed by DNase I digestion with MessageClean kit (GenHunter Corp., Nashville, TN). Total RNAs from human kidney, placenta, and cervical tumor were purchased (Clontech Laboratories, Palo Alto, CA), as were 0.5-kb polyadenylated RNAs for plant genes *Arabidopsis thaliana* chlorophyll a/b-binding protein (CAB) and RUBISCO activase (RCA) (Stratagene, La Jolla, CA). We synthesized a 1.8-kb polyadenylated transcript of peach ethylene receptor (ERS) using the AmpliScribe T3 High Yield Transcription kit (Epicentre Technologies, Madison, WI) and ERS cDNA cloned into pBluescript SK^+ (c75.MPD clone; a gift from Carole Bassett, USDA, Kearneysville, WV). We used UV spectrophotometry to quantify RNA, and formaldehyde gel electrophoresis [20] to evaluate RNA size and quality.

Table 3
Primers for synthesis of sRNA

Primer	Sequence (5′→3′)[a]	Reaction
P1	ATTCTAGAGGCCGAGGCGGCCGACATG $(T)_{29}$	Reverse transcription
P2-SP6	AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCC**ATTTAGGTGACACTATAGAAGGG**	Reverse transcription
P2-F	AAGCAGTGGTATCAACGCAGAGT	PCR
P1-R	ATTCTAGAGGCCGAGGCGGCCGACATG	PCR

[a] Underlined regions, *Sfi* I restriction enzyme recognition sites; Bold type, SP6 RNA polymerase binding site.

Complex sRNA synthesis

An overview of protocol for complex sRNA synthesis is presented in Figure 1. A directed RT reaction, followed by limited PCR results in a ds cDNA archive of the starting mRNA. The archived material is amplified and copied into sRNA with an IVT reaction.

Reverse transcription (RT) reaction

The RT reaction contained 1 μg of total RNA, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 75 mM KCl, 2 mM DTT, 1 mM each of dATP, dGTP, dCTP, dTTP, 1 μM each of primers P1 and P2-SP6 (Table 3), and 2 μl of Powerscript reverse transcriptase enzyme (Clontech Laboratories). A 10-μl mixture of total RNA and primers was heated for 5 min at 72°C, cooled on ice at least 2 min, and quickly spun before adding the remaining components (final volume 20 μl). The reaction proceeded for 1 h at 42°C and was terminated by heat inactivation at 70°C for 15 min. RT products were stored at −20°C until used.

Polymerase chain reaction

We used the Advantage 2 PCR kit (Clontech Laboratories) to synthesize ds cDNA from the RT products just mentioned (first-strand cDNA). The 100-μl reaction used 4 μl of RT product and 0.2 μM each of primers P2-F and P1-R (Table 3). The reaction used one cycle of 95°C for 20 s followed by 3–18 cycles of 95°C for 5 s and 68°C for 6 min. The PCR product was washed and concentrated to 20 μl using Centricon-100 columns (Millipore Corporation, Bedford, MA). Yield was determined using DyNA Quant 200 Fluorometer (Amersham Biosciences, Inc., Piscataway, NJ).

In vitro transcription (IVT) reaction

We used the AmpliScribe SP6 High Yield Transcription kit (Epicentre Technologies, Madison, WI) to prepare sRNA from 20–500 ng PCR products in a 20-μl reaction at 37°C for 4 h. After DNase I digestion, phenol-chloroform extraction, and ethanol precipitation, sRNA was pelleted, washed with 70% ethanol, resuspended in 20 μl diethyl pyrocarbonate-treated H_2O, and stored at −70°C until use. The sRNA was evaluated by denaturing agarose gel electrophoresis to estimate size and yield [20].

Real-time RT-PCR

Using either total RNA (direct RT) or sRNA (sRNA-RT), the RT portion of the real-time RT-PCR assay was carried out as described earlier, but the P1 and P2-SP6 primers were replaced with random hexamers (375 ng/reaction, Invitrogen Corp., Carlsbad, CA). We used the LightCycler (Roche Molecular Biochemicals, Indianapolis, IN) to conduct real-time PCR on 2 μl of 1:100 or 1:200 H_2O-diluted cDNA from the RT in a 20-μl reaction containing 2 μl of DNA Master SYBR Green mixture (includes

Table 4
Primers and real-time RT-PCR conditions for G3PDH and plant mRNAs

Gene name (GenBank accession code)	Sequence (5′→3′)[a]	Annealing/ acquisition temp. (°C)	Product size (bp)	PCR efficiency[b]
G3PDH (X01677)	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	58/86	450	1.95 ± 0.05
ERS (AY061640)	F: CACACCAAATGGCACCTCAA R: GATGGCATAATGGGCGACCTT	60/84	290	2.06 ± 0.11
CAB (X56062)	F: CTCAGGAATGGGCAGCACTACC R: CAGAATCCTACAAACGCCAACAGC	60/85	274	1.98 ± 0.01
RCA (X14212)	F: CAGGCTGCTTTGGGAGACG R: AAAAGGGAACAAAAGAGGACAAGA	60/85	282	1.94 ± 0.02

[a] *G3PDH* primers were obtained from Clontech. Other primers were designed using PrimerSelect of Lasergene Software (DNASTAR Inc., Madison, WI). F, forward primer; R, reverse primer.

[b] Mean ± SD of two determinations. PCR efficiency = $10^{[-1/\text{slope}]}$, where slope is given by LightCycler software from standard curves generated with 10-fold dilutions of direct RT using 1 μg of Caski total RNA spiked with 20 pg of each plant transcript [22].

Taq DNA Polymerase, dNTP, $MgCl_2$, and SYBR Green I dye), 0.16 μl of *Taq*Start Antibody (Clontech Laboratories), 0.4 μM of each gene-specific primer, and 4 μM $MgCl_2$. The optimal annealing and signal acquisition temperatures for each gene-specific primer pair was determined as described [21]. Primer sequences, annealing and signal acquisition temperatures, product length, and PCR efficiency are given in Table 4. Each LightCycler assay included duplicate reactions for each sample. Fluorescence curves were analyzed using the default fit point-arithmetic method of LightCycler software version 3.5. The noise band was set at the 0.1 level (log scale) to eliminate the background fluorescence. The crossing point (C_p) for each sample was expressed as mean $\pm$ SD. Melting curve analysis was conducted at the end of each LightCycler reaction to verify product specificity. In some cases, 10 μl of the reaction was subjected to 1.2% agarose gel electrophoresis with ethidium bromide staining.

Evaluation of sRNA synthesis

We spiked Caski total RNA with exogenous plant RNAs at different levels as shown in Table 2. Real-time RT-PCR of the cDNAs from direct RT was compared to that from cDNAs of the corresponding sRNA (sRNA-RT) to determine the amplification resulting from sRNA synthesis and to monitor the ability of sRNA to maintain relative proportions of specific mRNAs in starting total RNA.

Acknowledgments

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References

[1] S.J. Scheidl, et al., mRNA expression profiling of laser microbeam microdissected cells from slender embryonic structures, Am. J. Pathol. 160 (2002) 801–813.
[2] J.P. Carulli, et al., High throughput analysis of differential gene expression, J. Cell. Biochem. 30/31 (Suppl.) (1998) 286–296.
[3] S.D. Vernon, et al., Reproducibility of alternate probe synthesis approaches for gene expression profiling with arrays, J. Mol. Diagn. 2 (2000) 124–127.
[4] G. Brady, Expression profiling of single mammalian cells—small is beautiful, Yeast 17 (2000) 211–217.
[5] Y.Y. Zhu, E.M. Machleder, A. Chenchik, R. Li, P.D. Siebert, Reverse transcriptase template switching: a SMART™ approach for full-length cDNA library construction, Biotechniques 30 (2001) 892–897.
[6] A. Al-Taher, A. Bahein, T. Nolan, M. Hollingsworth, G. Brady, Global cDNA amplification combined with real-time RT-PCR: accurate quantification of multiple human potassium channel genes at the single cell level, Yeast 17 (2000) 201–210.
[7] R.N. Van Gelder, et al., Amplified RNA synthesized from limited quantities of heterogeneous cDNA, Proc. Natl. Acad. Sci. USA 87 (1990) 1663–1667.
[8] L. Luo, et al., Gene expression profiles of laser-captured adjacent neuronal subtypes, Nat. Med. 5 (1999) 117–122.
[9] E. Wang, L.D. Miller, G.A. Ohnmacht, E.T. Liu, F.M. Marincola, High-fidelity mRNA amplification for gene profiling, Nat. Biotechnol. 18 (2000) 457–459.
[10] J. Wang, L. Hu, S.R. Hamilton, K.R. Coombes, W. Zhang, RNA amplification strategies for cDNA microarray experiments, Biotechniques 34 (2003) 394–400.
[11] J. Phillips, J.H. Eberwine, Antisense RNA amplification: a linear amplification method for analyzing mRNA population from single cells, Methods 10 (1996) 283–288.
[12] U. Gubler, B.J. Hoffman, A simple and very efficient method for generating cDNA libraries, Gene 25 (1983) 263–269.
[13] W.O. Endege, K.E. Steinmann, L.A. Boardman, S.N. Thibodeau, R. Schlegel, Representative cDNA libraries and their utility in gene expression profiling, Biotechniques 26 (1999) 542–550.
[14] P. Gonzalez, J.S. Zigler, D.L. Epstein, T. Borras, Identification and isolation of differentially expressed genes from very small tissue samples, Biotechniques 26 (1999) 884–892.
[15] L.R. Baugh, A.A. Hill, E.L. Brown, C.P. Hunter, Quantitative analysis of mRNA amplification by in vitro transcription, Nucleic Acids Res. 29 (2001) e29.
[16] D.J. Lockhart, et al., Expression monitoring by hybridization to high-density oligonucloetide arrays, Nat. Biotechnol. 14 (1996) 1675–1680.
[17] L. Zhang, et al., Gene expression profiles in normal and cancer cells, Science 276 (1997) 1268–1272.
[18] H-M. Eun, Enzymology Primer for Recombinant DNA Technology, Academic Press, San Diego, 1996.
[19] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[20] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.
[21] M.S. Rajeevan, D.G. Ranamukhaarachchi, S.D. Vernon, E.R. Unger, Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies, Methods 25 (2001) 443–451; doi:10.1006/meth.2001.1266.
[22] M.W. Pfaffl, A new mathematical model for relative quantification in real-time PCR, Nucleic Acids Res. 29 (2002) 45e.