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Microarray-based comparison of three amplification methods for nanogram amounts of total RNA

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Singh, Ruchira, Rajanikanth J. Maganti, Sairam V. Jabba, Martin Wang, Glenn Deng, Joe Don Heath, Nurith Kurn, and Philine Wangemann. Microarray-based comparison of three amplification methods for nanogram amounts of total RNA. *Am J Physiol Cell Physiol* 288: C1179–C1189, 2005. First published December 22, 2004; doi:10.1152/ajpcell.00258.2004.—Gene expression profiling using microarrays requires microgram amounts of RNA, which limits its direct application for the study of nanogram RNA samples obtained using microdissection, laser capture microscopy, or needle biopsy. A novel system based on Ribo-SPIA technology (RS, Ovation-Biotin amplification and labeling system) was recently introduced. The utility of the RS system, an optimized prototype system for picogram RNA samples (pRS), and two T7-based systems involving one or two rounds of amplification (OneRA, Standard Protocol, or TwoRA, Small Sample Prototcol, version II) were evaluated in the present study. Mouse kidney (MK) and mouse universal reference (MUR) RNA samples, 0.3 ng to 10 μg, were analyzed using high-density Affymetrix Mouse Genome 430 2.0 GeneChip arrays. Call concordance between replicates, correlations of signal intensity, signal intensity ratios, and minimal fold increase necessary for significance were determined. All systems amplified partially overlapping sets of genes with similar signal intensity correlations. pRS amplified the highest number of genes from 10-ng RNA samples. We detected 24 of 26 genes verified by RT-PCR in samples prepared using pRS. TwoRA yielded somewhat higher call concordances than did RS and pRS (91.8% vs. 89.3% and 88.1%, respectively). Although all target preparation methods were suitable, pRS amplified the highest number of targets and was found to be suitable for amplification of as little as 0.3 ng of total RNA. In addition, RS and pRS were faster and simpler to use than the T7-based methods and resulted in the generation of cDNA, which is more stable than cRNA.

gene expression microarray analysis; microdissection; nucleic acid amplification techniques

MICROARRAYS PROVIDE A WIDELY accepted method for gene expression profiling on a genome-wide scale. Affymetrix GeneChip microarrays (Affymetrix, Santa Clara, CA) consist of oligonucleotide probes presented on a chip for hybridization to biotin-labeled cDNA or cRNA targets prepared from sample RNA (11). Hybridization is detected as fluorescence after binding to labeled streptavidin. Gene expression profiling using GeneChip arrays requires amplification of sample RNA, regardless of the available amounts of RNA. The Affymetrix standard protocol, which is well established for the preparation of cRNA targets from microgram amounts of total RNA, consists of one round of T7 amplification (OneRA) to generate biotin-labeled targets. Gene expression profiling of small tissue samples obtained by microdissection, laser capture microscopy, or needle biopsy that yield mere nanograms of RNA requires amplification of either the hybridization signal (8, 20) or the starting material. Methods for amplification of the starting material consist of exponential amplification by PCR (7, 13, 16, 22) and linear amplification with multiple rounds of T7 polymerase (1, 3, 4, 9, 15, 21). T7-based linear amplification systems have been used widely for amplification and production of cRNA targets. The Affymetrix small sample protocol consists of two rounds of amplification (TwoRA), including biotin labeling, and is a well-established, T7-based method for the preparation of cRNA targets from nanogram amounts of RNA (4). A novel linear amplification method, Ribo-SPIA, which enables mRNA amplification in a simple and rapid procedure, was recently introduced (NuGEN Technologies, San Carlos CA) (2, 5). In the present study, two systems based on this novel amplification technology, the Ovation Biotin System (RS) and a prototype system designed for target preparation from subnanogram level total RNA samples (picogram Ribo-SPIA prototype system, or pRS), were evaluated and compared with the T7-based one- and two-round amplification systems. The Ribo-SPIA systems have several advantages. Sample preparation can be performed within 1 day, which is much faster than the 20+ h required for TwoRA. Furthermore, RS and pRS yield cDNA, which is more stable than cRNA and eliminates biases in microarray results due to RNA degradation (19). The present study was designed to evaluate target populations generated by the Ribo-SPIA-based and T7-based systems. Targets were analyzed using high-density Affymetrix Mouse Genome 430 2.0 GeneChip arrays. Evaluation was based on direct comparisons and differential gene expression and included call concordances, signal correlations, and sensitivities. Detection of a limited set of targets was verified using RT-PCR.

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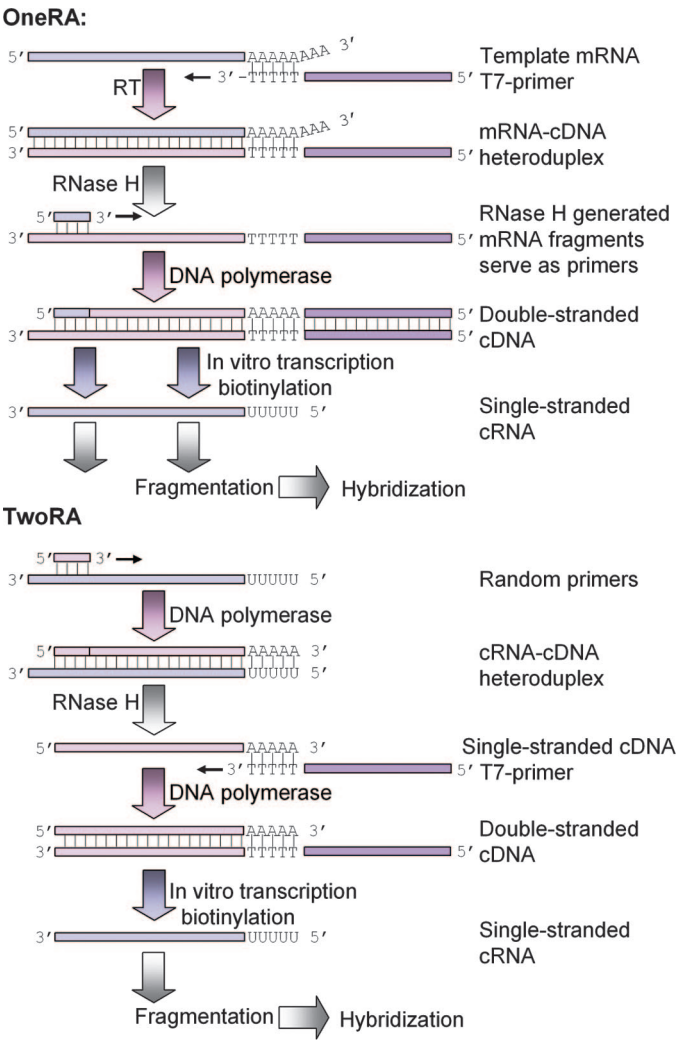

Fig. 1. Schematic representation of target preparation using one (OneRA) and two (TwoRA) rounds of amplification. RNA is shown in blue, and DNA is shown in pink.

MATERIALS AND METHODS

Target Preparation

Mouse kidney (MK) total RNA (Ambion, Austin, TX) and mouse universal reference (MUR) total RNA (Stratagene, La Jolla, CA) were chosen as sources for RNA to minimize biological variation and interexperimental differences. Targets were prepared using four different methods: *1*) one round of amplification (OneRA), *2*) two rounds of amplification (TwoRA), *3*) Ribo-SPIA linear amplification with the Ovation biotin system (RS), and *4*) Ribo-SPIA linear amplification with the picogram Ribo-SPIA prototype system (pRS).

One round of amplification. Starting with 10 μg of total RNA, cRNA targets were prepared using OneRA [standard protocol, version VII; Affymetrix (see https://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf)] (Fig. 1). Briefly, RNA was transcribed into cDNA using reverse transcriptase with a T7 primer that contains a promoter for DNA-dependent RNA polymerase (3, 21). After RNase H-mediated second-strand cDNA synthesis, the double-stranded cDNA (dscDNA) was purified and served as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction was performed in the presence of T7 RNA polymerase and a biotinylated nucleotide analog-ribonucleotide mix for cRNA amplification and biotin labeling. The biotinylated cRNA targets were then cleaned up, fragmented, and hybridized to GeneChip expression arrays.

Two rounds of amplification. Starting with 10 ng of total RNA, cRNA targets were generated using TwoRA (small sample protocol, version II; Affymetrix) (Fig. 1). Briefly, in vitro transcribed cRNA generated by the first round of amplification (see above) was subjected to a second round of amplification. cRNA was transcribed into cDNA using random primers and subsequently removed using heat-induced fragmentation. A second cDNA strand was synthesized using a T7-primer. A second cDNA strand was synthesized using a T7 primer to generate a dscDNA template containing the T7 promoter sequence. The resulting dscDNA was then amplified and labeled using a biotinylated nucleotide analog-ribonucleotide mix in the second IVT reaction. The labeled cRNA was then cleaned up, fragmented, and hybridized to GeneChip expression arrays.

Ribo-SPIA linear amplification. Ribo-SPIA-based RNA amplifications and target preparations were performed according to the manufacturer's instructions (picogram Ribo-SPIA prototype system, pRS and Ovation Biotin System, RS; see http://www.nugeninc.com/technology/index.shtml) (Fig. 2). Briefly, RNA was reverse transcribed into cDNA using reverse transcriptase with a DNA/RNA chimeric primer that is part DNA and part RNA. RNA was degraded by heating, and fragments served as primers for second-strand synthesis, yielding a dscDNA with an RNA/DNA heteroduplex at one

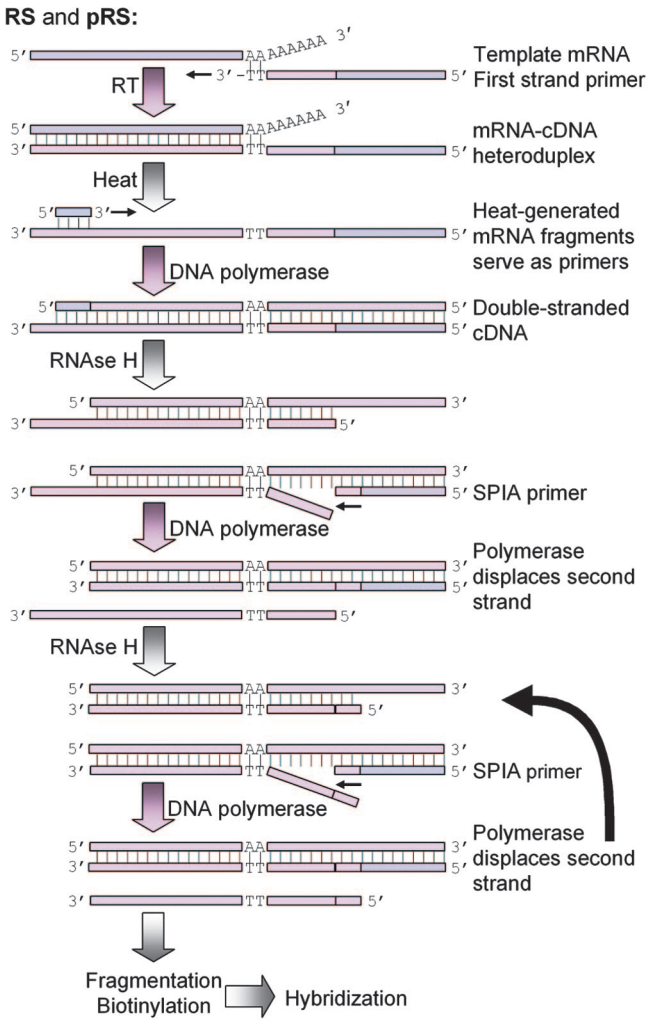

Fig. 2. Schematic representation of target preparation using Ribo-SPIA linear amplification (RS, Ovation-Biotin amplification and labeling system; and pRS, picogram RNA samples). RNA is shown in blue, and DNA is shown in pink.

Table 1. *GeneChip array quality metrics*

Method	No. of Chips	Scale Factor	Average Call All	%Present Call	Background Intensity	Noise	Raw Q
OneRA	6	3.5±0.6	696.5±7.8	45.4±4.0	42.6±2.0	2.6±0.2	1.4±0.1
TwoRA	6	2.4±0.2	698.0±7.8	46.7±2.5	47.2±2.8	3.0±0.2	1.5±0.1
RS	15	4.1±0.7	685.7±19.8	43.6±2.0	46.0±5.5	2.9±0.7	1.5±0.2
pRS	12	2.0±0.3	712.1±27.9	51.3±3.8	74.4±12.7	3.9±0.6	2.5±0.3

Values are averages ± SD. OneRA, one round of amplification; TwoRA, two rounds of amplification; RS, Ribo-SPIA technology; pRS, picogram RNA samples.

Table 2. *Verification of GeneChip array data obtained from MUR RNA*

Gene Symbol Affymetrix No.	Average Intensity				Left Primer Right Primer	Expected Product Size	Average Observed Product Size	RT-PCR Result
	OneRA	TwoRA	RS	pRS				
Cdknla	128	1252	302	1415	CCGTGGACAGTGAGCAGTT	286	279	Present
1421679_a_at	A	P	A	P	GAGTGCAAGACAGCGACAAG			
Nckl	584	826	117	205	TTTGGAACCATCTCCTCCAC	254	248	Present
1421487_a_at	A	P	A	P	GCCCAATGCAGTAAACAGTC			
Nme3	262	531	7	165	AAGCTGGTGGCACTGAAGCTAG	266	270	Present
1448905_at	A	P	A	P	CGAGTCGCTGCCATGAATTAC			
Cpsf1	3330	3990	184	827	GCCCATGCAAGAAAAGACAT	267	275	Present
1417665_a_at	P	P	A	P	GGTCTGTCTCCAGCCAAGTC			
Ruvb12	2858	2752	166	478	GGACCGCTTGCTCATTGTAT	275	276	Present
1422482_at	P	P	A	P	GTACTGTGTGGAGCGGGATT			
Hrmt111	1833	2311	48	243	ACCCACTGTGACATCCACAC	262	269	Present
1416844_at	P	P	A	*	GCATCAAGGACACCACTGAC			
Tce2	1980	1974	207	387	ATGTACCACGATTGGCGTTT	283	275	Present
1421971_a_at	P	P	A	P	CACAGGACCTCTCTGCCAGT			
Prkcsh	2563	1828	162	764	GACGAGCTGTAGCCTGAACC	287	279	Present
1416339_a_at	P	P	A	P	TCATCCATTTATCTCCCACCA			
1110035H23Rik	1210	1273	99	191	AGCAAAGGTGATTCCTGGTG	271	268	Present
1416315_at	P	P	A	P	CACTGGAGAGAGCTGATGGA			
Pold1	1480	1238	108	260	CTGCCCATCGACACTCAGTA	146	137	Present
1448187_at	P	P	A	P	GTCTTGCATCGTGTGTGGTC			
Nxph3	900	1082	136	68	ACTGAGGCTGAAATGGCAAC	257	256	Present
1419710_at	P	P	A	A	AGGTGGTTAGGGCAGAGGAT			
Nsd1	1132	882	111	438	GGAGTTCCAGTGCTCCTTCA	273	271	Present
1420882_a_at	P	P	A	P	GCCCATGTGTCCTCTGCTAT			
Slc22a5	677	667	101	180	GGGGAGTTTGTTTGTTTGTCA	291	298	Present
1450395_at	P	P	A	P	CACCGATGTGACACGACTG			
Lmnb2	334	313	1092	636	AACCTCCAGAACCCACCAG	251	243	Present
1448531_at	A	A	P	P	CACTCCGGGTAACCATCTCA			
Hspa4	343	146	1081	170	CAGTTGGGTGTAGGCATGGT	299	293	Present
1416147_at	A	A	P	P	GTCGGCAATGAGGTGTTCTT			
Neu3	83	22	536	539	GCCCTTATCCTTCCAATAATGC	294	297	Present
1419339_at	A	A	P	P	CAGTGTTAGAATGTCCCCCTGG			
Foxc2	898	355	3582	4382	GGGACCCCTAATGACTTCTG	124	123	Present
1416693_at	P	A	P	P	TTAAAGGCTCTGGGCAAGAA			
Hoxc4	357	275	2844	942	CCCCATCTCTTTCTTAGGC	271	259	Present
1422870_at	A	A	P	P	TAACCACGATGAGGGTAGGG			
Tle3	382	42	2146	1998	ACAGCGAGGATTTTCTGTGG	300	293	Present
1419654_at	P	A	P	P	AAAAGCACCCACACCAGTTG			
Max	215	151	2001	2268	TCCTCCCTCACCTCTGTTTG	197	185	Present
1423501_at	A	A	P	P	CTGCAAATCTGTCCCCACTT			
Dusp10	204	93	1889	2339	GACCTGGAACTGACTGCACA	138	138	Present
1417163_at	A	A	P	P	TAGCCTGTCTCCCCAGAGAA			
Tcp1-rsl	1051	326	1340	1110	ATCCTCTGGGAGCATCTGG	214	217	Present
1425195_a_at	P	A	P	P	GGTCCTCTGGTTTCCCTTTC			
Akl3	369	65	1304	795	ATTGCCTGCGTTTTCTTTTG	220	220	Present
1422078_at	P	A	P	P	CCTCTCACATCCATCCCTTG			
Hspa4	343	146	1081	170	CAGTTGGGTGTAGGCATGGT	299	294	Present
1416147_at	A	A	P	P	GTCGGCAATGAGGTGTTCTT			
Zfp235	70	66	889	645	AGCTGGTGATTGGCAGAAC	255	259	Present
1449329_at	A	A	P	P	TGGGTAGCACTATGCCTCAA			
Pik3rl	309	43	677	1289	TCATGTGTCAGAAGGCAGGA	287	290	Present
1425515_at	P	A	P	P	CCCAACCCTCCCACTTCTAT			

Targets were selected according to a set of criteria to ensure a fair comparison between TwoRA and RS (see MATERIALS AND METHODS). Targets called present or absent in all three replicates are marked P or A, respectively. Targets variably called present or absent are marked with an asterisk.

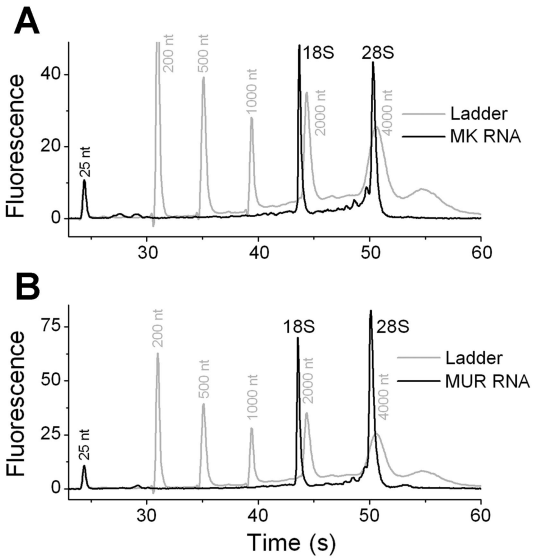

Fig. 3. Quality of RNA used as starting material. Electropherograms (Bioanalyzer, Agilent) of mouse kidney (MK) total RNA (*A*) and mouse universal reference (MUR) total RNA (*B*). Sharp peaks representing 18S and 28S rRNA demonstrate good quality (solid lines), although the mass ratio for the two rRNA species was lower for MK RNA. Each sample was spiked with a 25-nt marker to aid alignment of traces with a ladder consisting of 200-, 500-, 1,000-, 2,000-, and 4,000-nt fragments (shaded traces).

end. The RNA portion of the heteroduplex portion of the dscDNA was digested using RNase H added to the reaction mixture together with a DNA polymerase and a second chimeric cDNA/cRNA primer (amplification primer). Amplification was continued using primer extension, strand displacement, and degradation of the RNA portion of the primer extension product hybridized to the target to reveal part of the priming site for subsequent primer hybridization and extension by strand displacement DNA synthesis. Accumulated cDNA amplification products were fragmented and labeled to generate biotinylated cDNA targets. cDNA targets were prepared using RS starting with 100, 30, 10, or 3 ng of total RNA or pRS starting from 10, 3, or 0.3 ng of total RNA.

Quantification and Qualitative Assessment of RNA, cRNA, and cDNA

Quantities of total RNA, cRNA, and cDNA were determined using absorbance spectrophotometry (ND-1000 spectrophotometer; NanoDrop Technologies, Wilmington, DE). For total RNA and cRNA, the conventional conversion, 1 OD_{260} = 40 ng/μl, was used. For cDNA, the manufacturer's (NuGEN Technologies) recommended conversion, 1 OD_{260} = 33 ng/μl, was used. The quality of total RNA (eukaryotic total RNA nano assay) and cRNA and cDNA (mRNA smear nano assay) was determined using microfluidic electrophoresis (Bioanalyzer; Agilent Technologies, Palo Alto, CA).

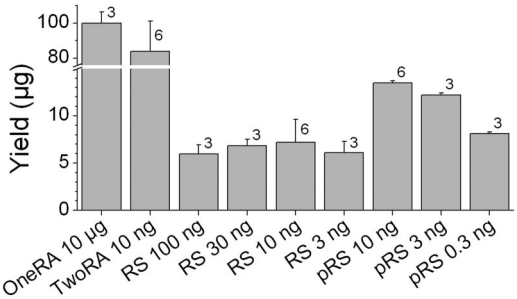

Fig. 4. Amplification yields. cRNA was obtained from 10 μg of total RNA using OneRA and from 10 ng of RNA using TwoRA. cDNA was obtained from 0.3, 3, 10, 30, or 100 ng of total RNA using RS and pRS. Numbers next to error bars represent the number of experiments conducted.

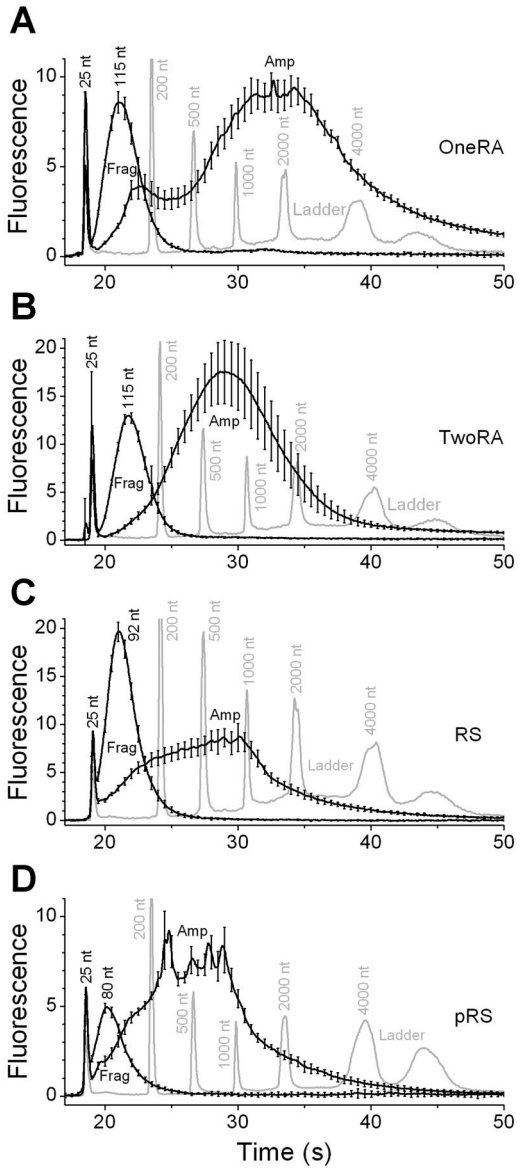

Fig. 5. Quality of cRNA and cDNA targets obtained after amplification (Amp) and fragmentation (Frag). *A*: averaged electropherograms of cRNA targets were prepared using OneRA from 10 μg of total RNA (n = 3). *B*: cRNA targets were prepared using TwoRA from 10 ng of total RNA (n = 3). *C*: cDNA targets were prepared using RS from 3, 10, 30, or 100 ng of total RNA (n = 1 each). *D*: cDNA targets were prepared using pRS from 10 ng of total RNA (n = 2). Each sample was spiked with a 25-nt marker to aid alignment of traces with a ladder consisting of 200-, 500-, 1,000-, 2,000-, and 4,000-nt fragments (shaded traces).

Microarray Analysis

Hybridizations of cRNA and cDNA targets were performed according to the manufacturer's recommended procedures on high-density oligonucleotide gene chips (Affymetrix Mouse Genome 430 2.0 GeneChip arrays; see http://www.affymetrix.com/support/technical/datasheets/mogarrays_datasheet.pdf). A total of 39 target prepara-

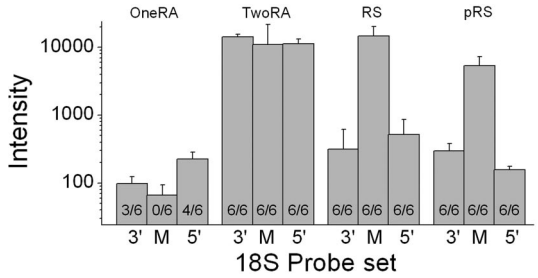

Fig. 6. Amplification of 18S rRNA. Signal intensity of the 3′, M, and 5′ probes sets. Probe sets were consistently called present for six target preparations prepared using TwoRA or using RS or pRS (each 6/6) but not for targets prepared using OneRA.

tions were performed, and each preparation was analyzed using one GeneChip array. Data were scaled to a target intensity of 500 (GCOS software; Affymetrix). Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values all were within acceptable limits (Table 1). Hybridization controls, BioB, BioC, BioD, and CreX, were called present on all chips and yielded the expected increases in intensities. Analyses of target populations were supported by GeneSpring (Silicon Genetics, Redwood City, CA), Excel (Microsoft, CA), and Origin 6.0 (OriginLab, Northampton, MA). Microarray data were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (accession nos. GSE1435 and GSE2019).

Call concordance. Probe sets were either called present (P), absent (A), or marginal (M) on the basis of detection P values (P, $P < 0.05$; M, $P = 0.05$–0.065; A, $P > 0.065$). Nine-field P/A/M matrices comparing data from two chips were constructed (Origin, version 6.0). Call concordance was the sum of the three concordant fields (P-P, A-A, and M-M), and call discordance was the sum of the other six fields (P-A, A-P, P-M, M-P, A-M, and M-A). Data were expressed as a percentage of the total number of probe sets ($n = 45{,}101$). Call concordance within a set of triplicate data was computed as average ± SD of three pairwise comparisons. Call concordance between two sets of triplicates was computed as average ± SD of all nine pairwise comparisons.

Signal correlation. Signal intensities without regard to present, absent, or marginal calls were correlated, and linear correlation coefficients (r) were determined (Origin, version 6.0). Correlation r values within a set of triplicates were reported as averages ± SD of the three possible correlations. Correlation r values between two sets of triplicates were based on averaged intensities and reported as a simple r value.

Sensitivity. Sensitivity was evaluated from the estimated fold change necessary for significance ($Fold_{sig}$). $Fold_{sig}$ was obtained according to $Fold_{sig} = [Avg + 2.776 \times \sqrt{(2/3 \times SD^2)}]/Avg$, where Avg and SD are the average and standard deviation of the signal intensity of genes called present in all replicates, and 2.776 is the t value at $P = 0.05$, assuming 4 df.

Differential gene expression. The signal intensity ratios of genes detected in all targets prepared from 10 μg and 10 ng MK and MUR RNA using OneRA, TwoRA, RS, and pRS were calculated from the averages of triplicates. Ratios were correlated between amplification systems, and correlation coefficients were obtained.

Verification of Microarray Data

A small number of gene targets amplified from MUR RNA and detected using microarrays were verified by performing RT-PCR (Table 2). Targets were chosen on the basis of gene array data obtained from 10 ng of MUR RNA amplified using either TwoRA or RS. For each method, 13 targets were selected on the basis of six criteria established to ensure fair selection between these two methods. *1*) Targets must be called present in three replicates of the considered amplification method (TwoRA or RS). *2*) Targets must be called absent in three replicates of the other amplification method. *3*) Hybridization intensities of the three replicates of the considered amplification method must be >100. *4*) Targets of the considered amplification method must be annotated by Affymetrix as full-length mRNA and not as expressed sequence tag. *5*) Hybridization intensities between sets of chosen targets must be comparable. *6*) Fold differences between called present using one amplification method and absent using the other method must be comparable between sets of chosen targets. Average intensities and calls for the 26 selected genes are shown in Table 2, not only for TwoRA and RS but also for OneRA and pRS.

Real time RT-PCR was performed in the presence of 0.2× SYBR Green I (Molecular Probes, Eugene, OR) using MUR RNA and gene-specific primers (Table 1) (23). RT was performed for 30 min at 50°C and for 15 min at 95°C. PCR consisted of 50 cycles of 1 min at 60°C, 1 min at 72°C, 7-s heating to hot measurement temperature, 13-s hot measurement at 78°C below product melting temperature, and 1 min at 94°C. Hot measurements were performed to eliminate the detection of primer dimers that tend to have melting temperatures between 72 and 75°C. PCR was followed by melting (60–95°C). Targets were considered present when a single product of the appropriate size was obtained. Target sizes were determined by performing microfluidic electrophoresis (Bioanalyzer; Agilent Technologies). The average difference between observed and expected product sizes was 5.6 ± 3.8 bp ($n = 25$).

Statistics

Gene array experiments were performed in triplicate. RT-PCR experiments were performed at least in duplicate, mostly in quadruplicate. Data, including quality control values, are expressed as averages ± SD. The significance of continuous data was determined using one-way ANOVA with a Bonferroni post hoc test. Significance was assumed at $P < 0.05$.

RESULTS

Target Yields and Amplification

Samples of total RNA ranging from 0.3 ng to 10 μg were used as starting materials. Quantities were verified spectrophotometrically. RNA qualities were evaluated by performing

Table 3. *Amplification-induced 3′ bias measured by signal intensity on GeneChip arrays*

Gene	Ratio	Distances from 3′	OneRA (10 μg)	TwoRA (10 ng)	RS (10 ng)	pRS (10 ng)
GAPDH	3′/M	387/770	0.87±0.03	1.09±0.04*	1.6±0.1*	1.12±0.02*
GAPDH	3′/5′	387/1,138	0.80±0.03	1.7±0.1*	1.5±0.1*	1.12±0.03*
β-Actin	3′/M	972/1,298	1.09±0.05	1.2±0.1	2.9±0.2*	2.5±0.1*
β-Actin	3′/5′	972/1,665	1.17±0.06	1.5±0.1*	3.1±0.1*	3.8±0.2*
Transferrin receptor	3′/5′	528/2,225	3.4±0.2			

Values are averages ± SD. Ratios were obtained for targets that were prepared from MUR RNA (each $n = 3$) by one (OneRA), or two (TwoRA) rounds of amplification or by Ribo-SPIA linear amplification (RS and pRS). OneRA was taken as standard, and significantly different data are marked with an asterisk.

Table 4. *Call distribution, call concordance, signal correlation, and estimated average fold change necessary for significance*

Method	RNA	P, %	A, %	M, %	Concordance, %	Correlation, *r*	$Fold_{sig}$
OneRA	10 μg MK	42.0±2.1	56.1±2.1	1.94±0.06	90.8±0.6	0.991±0.005	1.27±0.20
TwoRA	10 ng MK	44.5±1.3	53.5±1.2	1.96±0.06	91.4±0.4	0.99562±0.00004	1.26±0.19*
RS	100 ng MK	45.8±0.5*	52.4±0.5*	1.81±0.11	89.7±0.3*	0.991±0.001	1.33±0.22*
RS	30 ng MK	45.2±0.7*	53.0±0.7*	1.75±0.02	90.0±0.3	0.995±0.001	1.29±0.21*
RS	10 ng MK	41.7±1.0	56.5±1.0	1.78±0.05	88.4±0.6*	0.981±0.010	1.44±0.28*
RS	3 ng MK	41.2±0.2	57.1±0.3	1.77±0.14	88.5±0.2*	0.990±0.001	1.43±0.30*
pRS	10 ng MK	50.4±0.7	48.2±0.7	1.37±0.06	88.4±0.04*	0.9957±0.0002	1.39±0.34*
OneRA	10 μg MUR	48.8±0.6	49.3±0.6	1.87±0.03	92.2±0.1	0.9978±0.0002	1.20±0.17
TwoRA	10 ng MUR	48.8±0.5	49.2±0.5	1.97±0.04	91.8±0.2	0.9964±0.0003	1.24±0.18*
RS	10 ng MUR	44.2±0.7*	53.8±0.7*	1.91±0.06	89.3±0.3*	0.995±0.001	1.29±0.21*
pRS	10 ng MUR	54.8±1.1	43.7±1.0	1.43±0.12	88.1±0.1*	0.991±0.005	1.40±0.30*
pRS	3 ng MUR	53.2±0.9	45.3±0.9	1.43±0.12	87.0±0.3*	0.990±0.003	1.50±0.39*
pRS	0.3 ng MUR	45.9±1.2	52.9±1.2	1.27±0.06	83.6±0.8*	0.966±0.005*	1.91±0.64*

Call distributions and call concordances are relative to the total number of 45,101 probe sets. Call concordances and signal correlations are based on the three possible comparisons within triplicate samples. Estimated average fold changes necessary for significance ($Fold_{sig}$) were based on average intensity and standard deviation for present genes. Targets were prepared from mouse kidney (MK) or mouse universal reference (MUR) RNA using OneRA or TwoRA or Ribo-SPIA linear amplification (RS or pRS). Targets prepared using OneRA from 10 μg of RNA were taken as standard, and significantly different data are marked with an asterisk.

microfluidic electrophoresis (Fig. 3). MUR RNA was of slightly higher quality than MK RNA as evidenced by the larger ratio between the 28S and 18S peak. cRNA targets were prepared using OneRA or TwoRA starting with 10 μg or 10 ng RNA, respectively. Alternatively, cDNA targets were prepared using RS starting with 3, 10, 30, or 100 ng of RNA and using pRS starting with 0.3, 3, and 10 ng of RNA. Figure 4 summarizes cRNA and cDNA target yields obtained after amplification. Yields for cRNA and cDNA targets were significantly different, but each was sufficient for preparation of the hybridization cocktail (Affymetrix). The size distributions of amplified products and of biotinylated and fragmented targets are shown in Fig. 5.

These target preparation methods, OneRA, TwoRA, RS and pRS, are designed to amplify mRNA by priming at the polyA tail. Adherence to this design goal was evaluated using gene array analysis. PolyA tail-independent amplification was evaluated by probes for 18S rRNA, which is thought not to contain a polyA tail and therefore should not be amplified and consequently not called present. Probe sets for 18S hybridized with targets prepared using OneRA yielded low signal intensities and were not consistently called present (see Fig. 6). In contrast, 18S probe sets hybridized with targets prepared using TwoRA, RS, or pRS were consistently called present. Hybridization with targets prepared using TwoRA, RS, or pRS yielded high signal intensities.

Bias toward amplification of the 3′ end of targets was evaluated using probes located toward the 3′ and 5′ ends and in the middle of GAPDH, β-actin, and transferrin receptor. Signal intensity ratios, 3′-5′ and 3′-M, were calculated (Table 3). Ratios should ideally be unity. Ratios for targets prepared using OneRA were closest to unity. Increased 3′-5′ ratios were noted for all other target preparation methods from the lower-input total RNA samples. This observation is similar to that noted by others and likely does not affect the quality of expression profiling with the GeneChip arrays, because the probe design for these arrays is 3′ biased (12). 3′ Biases for targets prepared using RS and pRS were similar, regardless of the amount of starting material.

The number of probe sets that were called present, absent, or marginal were tabulated (Table 4). pRS had the highest number of present calls, followed by OneRA, TwoRA, and RS, respectively. More genes were consistently (in all three replicates) called present after pRS than after TwoRA or RS (Fig. 7). A sizable number of targets (n = 13,539, based on MUR RNA) was amplified by all three methods. Similar observations were

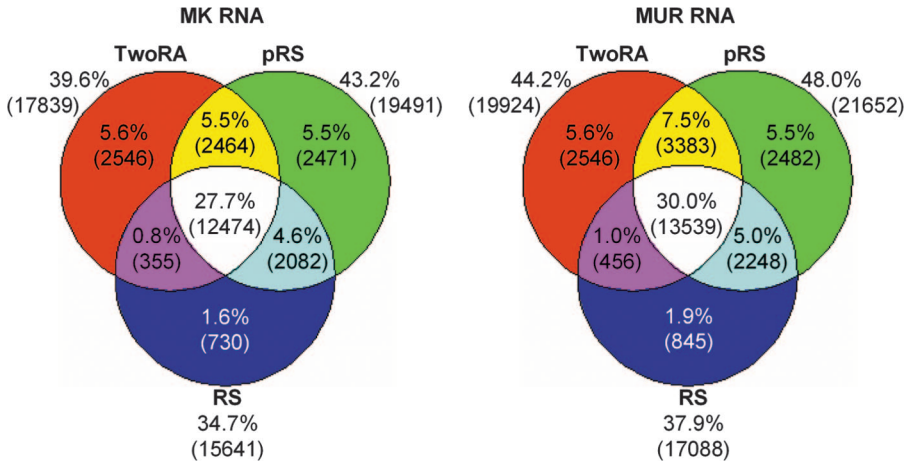

Fig. 7. Venn diagrams of genes consistently called present. Target populations were prepared from 10 ng of MK or MUR RNA using TwoRA or using RS or pRS. The total of 45,101 probe sets is 100%.

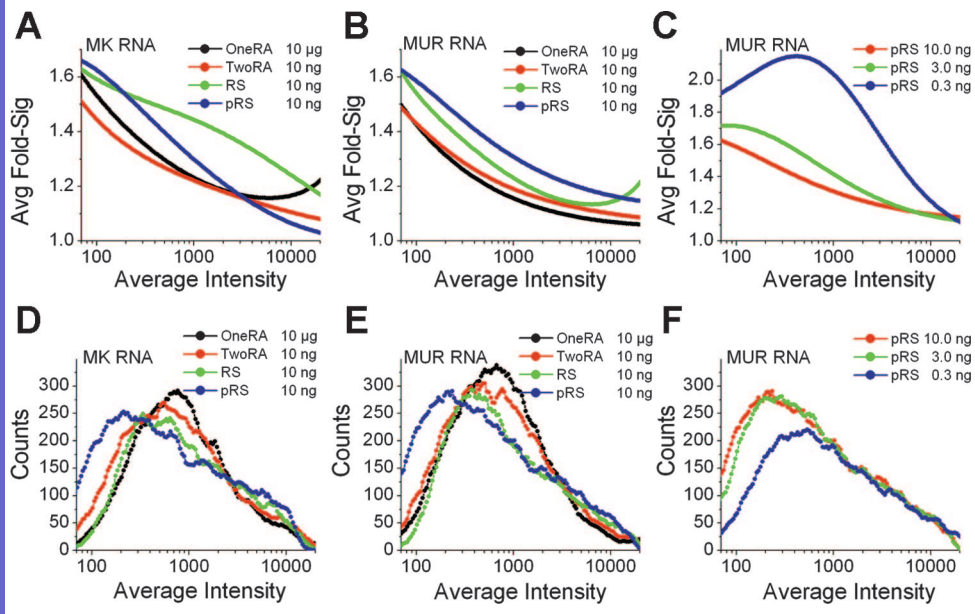

Fig. 8. Intensity dependence of estimated fold change necessary for significance. Fold changes for significance were computed on the basis of averages and standard deviations. Traces represent smoothed averages. *A*, *C*, and *E*: estimated fold changes for significance computed from target populations amplified from different amounts of MK RNA or MUR RNA. *B*, *D*, and *F*: smoothed frequency distributions of intensities are provided for comparison. Target populations were prepared using OneRA or TwoRA or using RS or pRS.

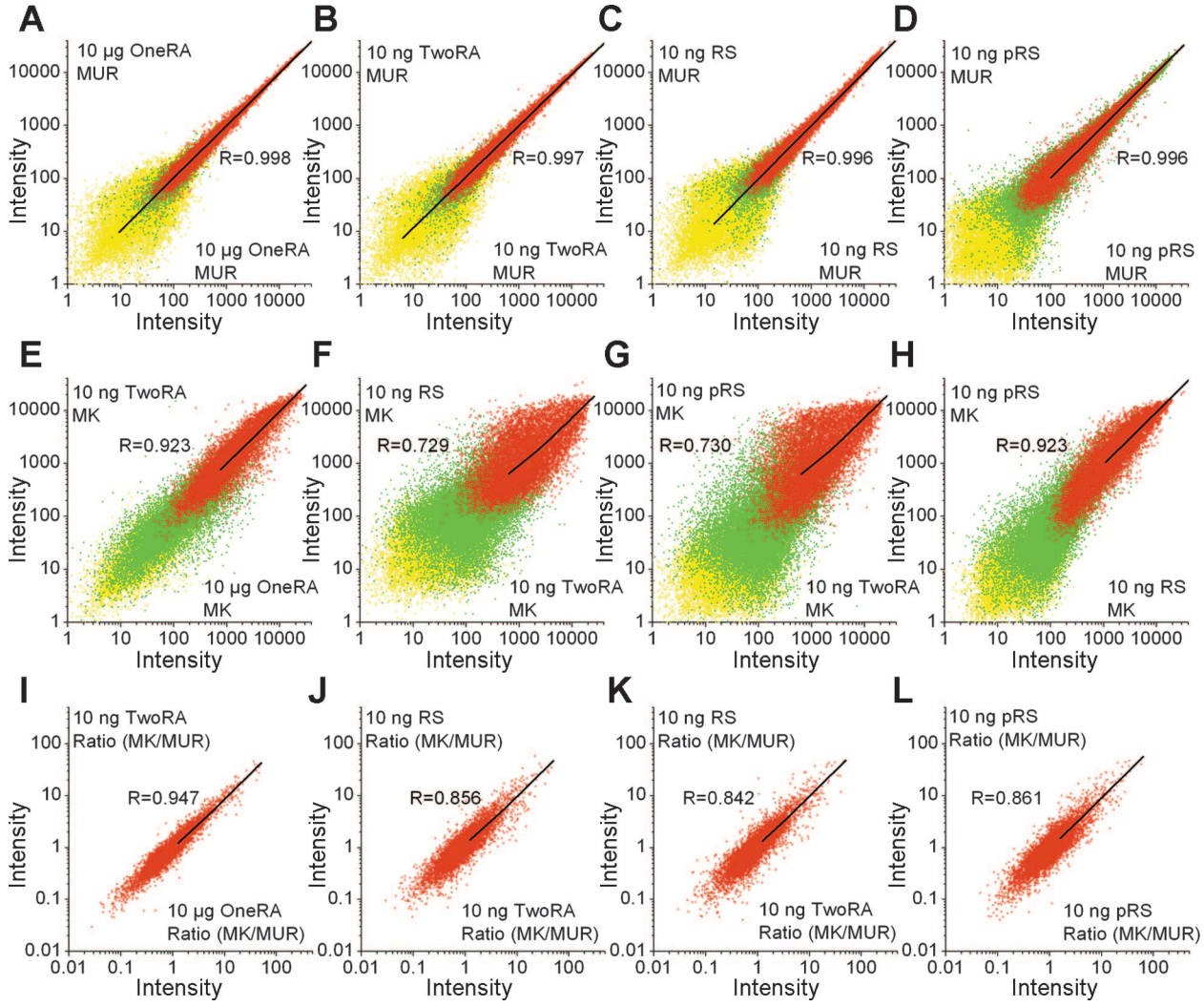

Fig. 9. Correlation of signal intensities and ratios. *A–D*: best examples of correlations of individual data sets (replicates) prepared using one OneRA or TwoRA or using RS or pRS from MUR RNA. *E–H*: correlation of averaged data sets prepared by OneRA, TwoRA, RS, or pRS from MK RNA. *I–L*: correlation of ratios computed from averaged data sets (MK/MUR) that originated from samples prepared using OneRA, TwoRA, RS, or pRS.

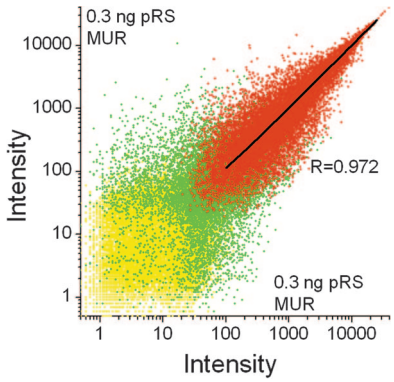

Fig. 10. Correlation of signal intensity for 0.3 ng of total RNA. Best example of correlations of individual data sets (replicates) prepared using pRS from MUR RNA.

made for MK RNA. For targets prepared using RS and pRS, present calls decreased and absent calls increased with decreasing amounts of starting material (Table 4). Sensitivity was computed as the average fold change necessary for significance (Table 4). $Fold_{sig}$ was found to be intensity dependent (Fig. 8).

Verification of Amplification

A sizable number of targets (n = 13,995; 13,539 + 456, based on MUR RNA) were amplified from 10 ng of RNA using both TwoRA and RS (Fig. 7). An additional 5,929 (2,546 + 3,383) targets were TwoRA specific because they were amplified using TwoRA and not RS, and an additional 3,093 (845 + 2,248) targets were RS specific because they were amplified using RS and not TwoRA. TwoRA- and RS-specific targets had similar distributions of hybridization intensities and of fold differences between hybridization intensities that were called present and called absent. TwoRA- and RS-specific target populations were sampled according to a set of criteria (see MATERIALS AND METHODS) that ensured fair comparison between these two systems. All 13 TwoRA-specific and all 13 RS-specific targets could be verified in MUR RNA (Table 1), a finding consistent with equal fidelity of TwoRA and RS. Of the 26 selected targets, 15 were called present using OneRA and 24 were called present using pRS. Quantitative comparisons other than between TwoRA and RS are not warranted, given that unbiased selection was ensured only for TwoRA and RS. Taken together, these data demonstrate equal fidelity of TwoRA and RS and suggest that each amplification system amplifies a unique set of targets in addition to the overlapping target sets.

Reproducibility between Replicates

Call concordances and correlation coefficients within triplicate samples were calculated to evaluate reproducibility between replicates (Table 4; the best examples are shown in Figs. 9, *A–D*, and 10). Between replicates, call concordances were similar for targets prepared using OneRA or TwoRA and somewhat lower for RS and pRS (92.2, 91.8, 89.3, and 88.1%, respectively, for target prepared from 10 μg and 10 ng of MUR RNA). Signal correlations were similar for targets prepared using all amplification systems.

Fidelity of Amplification Systems

Direct comparison. Call concordances were obtained from comparisons of samples prepared from 10 μg of RNA using OneRA and from 10 ng using TwoRA, RS, or pRS (Table 5). Call concordances between OneRA and TwoRA were higher than for any other comparison. Observations were similar for MK and MUR RNA. Signal intensities were correlated between amplification systems (Fig. 9, *E–H*). Correlation coefficients between OneRA and TwoRA and between RS and pRS were better, most likely because of the greater similarities between these amplification systems. Low call concordances and signal correlations between T7- and Ribo-SPIA-based systems suggest that the two amplification methods may introduce different biases or that cDNA and cRNA perform differently on GeneChip arrays. Indeed, cDNA/DNA hybridizations may be more reliable than cRNA/DNA hybridization because of the lesser complexity of cDNA/DNA interactions (17, 18, 24), which may affect both present vs. absent calls and signal intensities.

Differential gene expression. Amplification-induced bias in signal intensity can be canceled by computing differential gene expression. Signal intensity ratios of data originating from MK and MUR RNA were calculated from averages of triplicates.

Table 5. *Call concordances and signal intensity correlations between systems*

RNA	Concordance, %	P-P, %	A-A, %	A-P, %	P-A, %	Correlation, r
MK RNA						
OneRA-TwoRA	86.7±0.8	37.6±1.3	49.0±0.9	5.9±1.3	3.7±0.6	0.923
OneRA-RS	76.5±1.0	31.2±1.0	45.3±1.1	9.8±1.1	10.1±0.9	0.693
OneRA-pRS	60.7±0.3	27.4±1.0	33.2±0.9	22.0±1.1	14.1±0.8	0.702
TwoRA-RS	77.5±0.4	32.9±0.6	44.5±0.8	8.1±0.7	10.8±0.6	0.729
TwoRA-pRS	60.6±0.2	28.6±0.7	31.9±0.6	20.8±0.7	15.3±0.6	0.730
RS-pRS	60.0±0.3	26.9±0.5	33.1±0.7	22.6±0.5	14.3±0.6	0.923
MUR RNA						
OneRA-TwoRA	87.6±0.2	43.5±0.3	43.9±0.4	4.4±0.3	4.5±0.3	0.930
OneRA-RS	74.0±0.3	34.6±0.4	39.3±0.4	8.9±0.3	13.4±0.5	0.685
OneRA-pRS	59.3±0.1	32.6±0.3	26.7±0.2	21.8±0.3	15.6±0.3	0.734
TwoRA-RS	75.3±0.3	35.3±0.5	40.0±0.3	8.3±0.3	12.7±0.5	0.701
TwoRA-pRS	58.7±0.2	32.3±0.3	26.4±0.2	22.0±0.2	15.9±0.2	0.741
RS-pRS	56.6±0.2	28.9±0.4	27.6±0.3	25.4±0.4	14.8±0.2	0.907

Call concordances are calculated between target populations generated from 10 μg of RNA using one (OneRA), or from 10 ng RNA using two (TwoRA), rounds of amplification or Ribo-SP1A linear amplification (RS or pRS). Call concordances are averages ± SD of the nine possible comparisons between two sets of triplicates. Signal correlation coefficients were obtained from correlation of averaged triplicates.

Ratios of samples that were prepared using different amplification systems were compared. Correlation coefficients were obtained for probes that were called present in all sets of triplicates prepared using OneRA, TwoRA, RS, and pRS (Fig. 9, *I–L*). Correlation between TwoRA and OneRA was considerably better than between other amplification systems.

DISCUSSION

Microarray-based gene expression profiling of microdissected tissue samples and needle biopsies is often limited because of insufficient amounts of RNA. Methods devised to overcome this limitation include signal amplification (8, 20) and sample amplification, including exponential amplification using PCR (7, 9, 13, 16, 22) and linear amplification using T7 polymerase (1, 4, 15, 21). T7-based linear amplification methods, including TwoRA, are commonly used for preparation of targets from nanogram amounts of RNA (3, 21). RS and pRS are novel systems that are based on Ribo-SPIA technology that was recently introduced as an alternative to TwoRA. The goal of the present study was to compare RS, pRS, TwoRA, and OneRA. Target yields and qualities, amplification biases, call concordances, correlation coefficients, and sensitivities were determined. Evaluations were based on direct comparisons and on differential gene expression. OneRA was taken as the baseline, mostly for historical reasons (11). Although OneRA is the major linear amplification protocol used on the Affymetrix platform, it should not be assumed to generate a "true" or "standard" gene expression profile as implied by its commercial name.

RS and pRS generated significantly smaller amounts of cDNA than TwoRA generated cRNA (Fig. 4), although all systems produced sufficient amounts of targets, given that 15 μg of cRNA and only 2 μg of cDNA are needed for array hybridization. Yields of cDNA targets prepared using RS and pRS were independent of the amount of starting material, which is similar to the pattern observed with PCR.

Probe sets for 18S rRNA were low in signal intensity and inconsistently called present in targets prepared using OneRA. However, 18S rRNA was consistently called present, with high signal intensities in target preparations prepared using TwoRA, RS, and pRS (Fig. 6). This observation may indicate that both T7- and Ribo-SPIA-based amplification systems prime at internal polyA sites. Alternatively, it is conceivable that polyadenylated forms 18S rRNA may be present as recently observed in yeast (10). Thus it is possible that the higher sensitivity afforded by RNA amplification enables the detection of small amounts of polyadenylated rRNA on the arrays. The majority of amplified transcripts generated using RS and pRS were up to 1,000 nt in length (Fig. 5). A similar length distribution was found for TwoRA. In contrast, the majority of amplified transcripts generated using OneRA were up to 2,500 nt in length. These differences are not likely to be important on Affymetrix arrays, in which >98% of the probe sets are located <600 nt from the 3′ end.

3′ Bias was further determined for three genes with probe sets that varied in their distances from the 3′ end of the mRNA (Table 3). 3′/M and 3′/5′ ratios for GAPDH are best suited to predict fidelity of expression profiling given that >98% of probe sets on Affymetrix Mouse Genome 430 2.0 GeneChips are specific for sequences within 600 nt from the 3′ end. The observation that 3′-M and 3′-5′ ratios for GAPDH were close to unity is consistent with the view that TwoRA, RS, and pRS amplify targets with high fidelity. The observation that 3′-5′ ratios for the transferrin receptor, which are based on probe sets located 528 and 2,225 nt from the 3′ end, could be obtained only for OneRA is consistent with the finding that OneRA yielded longer transcripts than TwoRA, RS, or pRS.

The RNA starting material for MK and MUR differed notably in quality (Fig. 3). Consistently, significant differences between MK and MUR RNA were found for probe sets that are

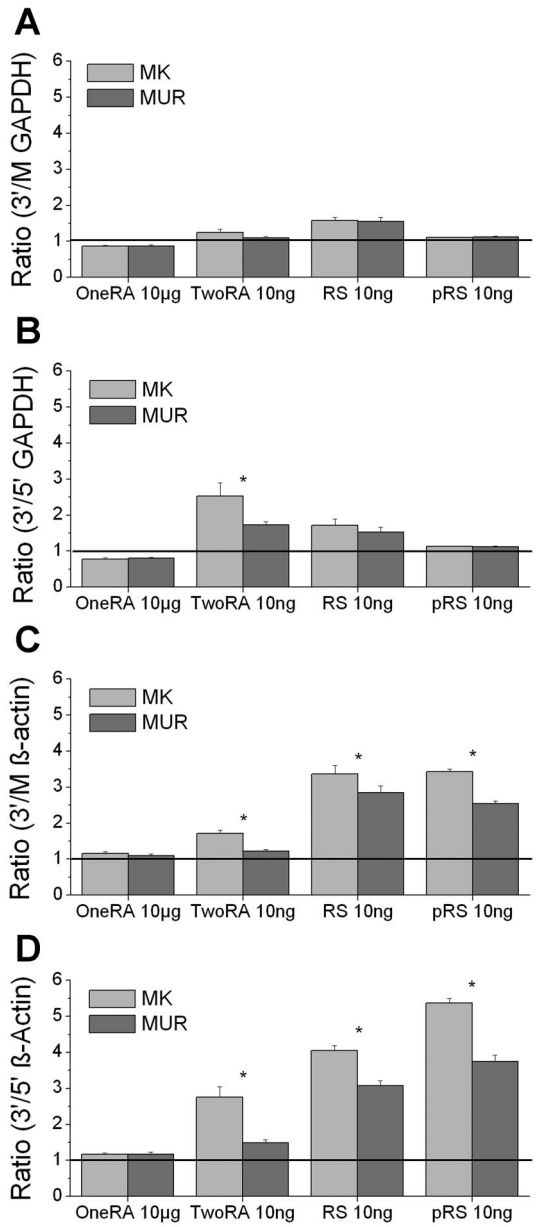

Fig. 11. 3′ Bias as a function of the quality of the RNA starting material. MK RNA was of slightly lesser quality than MUR RNA as evidenced by the lesser ratio of the 18S and the 28S peaks (see Fig. 3). Targets were prepared using OneRA from 10 μg of RNA, TwoRA from 10 ng of RNA, or RS or pRS from 10 ng of RNA. *A–D*: 3′-M and 3′-5′ ratios for GAPDH and β-actin; $n = 3$ for each ratio. *Significant differences between MK and MUR RNA. Note that no significant differences were found for OneRA or for ratios that spanned short distances.

located 972 and 1,665 nt from the 3′ end (3′-5′ ratio for β-actin; Fig. 11). However, no significant differences were found for ratios of probe sets located 387 and 770 nt from the 3′ end (3′-M ratio for GAPDH; Fig. 11). Given that >98% of probes are located within 600 nt of the 3′ end, the difference in RNA quality was not expected to have a major effect on the gene array data. Consistently, analyses of GeneChip array data from both RNA gave similar results.

A sizable number of targets (13,539 in 10 ng of MUR RNA) were detected using TwoRA, RS, and pRS. The highest number of genes (21,652 in 10 ng of MUR RNA) was consistently called present in targets prepared using pRS compared with RS and TwoRA (Fig. 7). pRS consistently amplified 15,179 targets from 0.3 ng of total RNA with acceptable reproducibility as measured by call concordance, signal correlation, and sensitivity (Table 4 and Figs. 8 and 10). Verification of 13 TwoRA- and 13 RS-specific targets using RT-PCR (Table 2) demonstrated that TwoRA and RS amplified partially unique sets of targets.

Reproducibility between replicates was based on call concordance and estimated fold changes necessary for significance (Table 4 and Fig. 8). Reproducibility for targets that were prepared from 10 ng of RNA using TwoRA was higher than for targets prepared from 10 ng of RNA using RS or pRS. Fold changes necessary for significance, however, were well below 2 for all amplification systems. A fold change of 2 is sometimes used as an arbitrary lower limit for significance.

As expected, comparisons of targets populations prepared using different amplification methods yielded poorer call concordances and signal intensity correlations than comparisons of targets populations prepared using the same method (9, 14). This difference illustrates the presence of system-specific biases.

Call concordances were higher between the two T7-based systems, OneRA and TwoRA, than between T7- and Ribo-SPIA-based systems (Table 5), most likely because of the greater similarity of the methods and the similar number of present calls. Although RS and pRS are similar methods, they differed greatly in the number of present calls, which resulted in poorer call concordance. Signal correlation coefficients within the two T7-based (OneRA and TwoRA) or within the two Ribo-SPIA-based methods (RS and pRS) were significantly higher than any comparison between these methods (Table 5 and Fig. 9, *E–H*). The observation that absent vs. present calls between OneRA and TwoRA or between OneRA and pRS were significantly larger than present vs. absent calls is consistent with amplification of rare messages using TwoRA and pRS. Amplification is likely to raise signal intensities of rare genes above the noise level (6).

Differential gene expression was computed in an attempt to cancel system-dependent biases. Nevertheless, correlation between ratios of targets prepared using T7-based systems, TwoRA and OneRA, was considerably better than that between ratios of T7- and Ribo-SPIA-based methods (Fig. 9, *G–I*). This observation underscores the presence of system-specific biases.

All nanogram amplification methods, TwoRA, RS, and pRS, yielded sufficient material for gene array work, although TwoRA yielded quantitatively more cRNA than RS or pRS yielded cDNA. The RS and pRS target preparation methods produced results comparable to those observed using more traditional T7-based methods and will enable studies of smaller RNA samples because the required input level is lower and the time and effort required for amplification are lower. pRS reproducibly amplified the highest number of targets and was found to be suitable for amplification of total RNA from amounts as low as 0.3 ng. Reproducibility and sensitivity of TwoRA relative to OneRA were higher than those of RS or pRS. All amplification systems, OneRA, TwoRA, RS, and pRS, amplified large overlapping sets of targets. Target preparations using RS and pRS were faster and produced cDNA, which is more stable than cRNA and thus can be banked for additional studies. The presence of system-specific biases prompts the recommendation that changes in amplification methodology within a study be avoided. Indeed, in the anticipation of future refined studies on nanogram amounts RNA, investigators may want to choose a nanogram amplification system for a pilot study even if microgram amounts of RNA are available.

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DISCLOSURES

J. D. Heath and N. Kurn are employees of NuGEN Technologies, Inc. M. Wang and G. Deng were employees of NuGEN at the time this study was conducted. NuGEN has patents and patents pending on some of the products and processes described in this article.

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