regions in genome sequencing projects (4–6). The method described here has limited applicability to these situations because one must know the sequence of the inverted repeats to design a blocking oligonucleotide. In such circumstances, it would be preferable to separate the inverted repeats by cloning them separately or to increase the distance between the inverted repeats by inserting a transposon (of known sequence) into the loop of the hairpin (7). Alternatively, one could design sequencing oligonucleotides with higher melting temperatures and attempt to minimize hairpin formation by raising the annealing temperature in each sequencing cycle. For sequencing Gateway entry clones using standard forward and reverse primers, the blocking oligonucleotide method described here is a less expensive and easier way to acquire long sequence reads.

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DNase I treatment of cDNA first strands prevents RT-PCR amplification of contaminating DNA sequences

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Retropseudogenes arise in evolution by reverse transcription of processed mRNAs and incorporation of the resulting cDNAs back into the genome (1). They are intronless DNA sequences that share a high degree of homology with the cDNA of their corresponding active genes. The human genome is estimated to contain approximately 23,000 to 33,000 retropseudogenes (2). An example of a gene family exhibiting retropseudogenes is the high mobility group protein family of which the *HMGA1* (HMGA protein family) and *HMGB1* (HMGB protein family) genes have been shown to contain many retropseudogenes (3–5). Several retropseudogenes have also been described for the housekeeping genes *GAPDH* and β -actin (*ACTB*) (6,7). In a lot of cases, the sequence homology of the retropseudogenes compared with the functional mRNA can be so high that retropseudogenes may serve as an appropriate template in a PCR using genomic DNA and a primer set detecting a cDNA sequence of the active gene (4,8).

Unfortunately, PCR products amplified from retropseudogenes cannot be distinguished in size from those amplified from cDNA, as retropseudogenes lack introns. A similar problem exists for specific amplification of intronless genes because in a RT-PCR, discrimination of cDNA and DNA amplification products by differing sizes of PCR products is not possible. Although the proportion of intronless human genes is generally thought to be no higher than 5% of all genes, there do exist some families of intronless genes like the histone genes and the G-protein-coupled receptor (GPCR) gene family (9).

Thus, genomic DNA (gDNA) contamination of cDNAs affects specific amplification of both housekeeping or target genes exhibiting at least one retropseudogene and intronless genes. Accordingly, a gDNA-free cDNA is needed to perform reliable RT-PCR analyses. Herein we describe an improvement of the standard method to eliminate detectable gDNA contamination in cDNA preparations.

Table 1. Reaction Conditions for RT-PCR (*GAPDH*, *ACTB*, *HMGB1*, and *HMGA1*) and PCR (*HMGA2*, *HMGN2*, and *HMGB1*) Analyses in This Study

Gene	Name of Primer	Sequence of Primer	Volume of DNase cDNA Used as a Template (μL)	Annealing Temperature (°C)	Size of PCR Fragment (bp)
<i>GAPDH</i> (expression)	GAPDH2.up	5'-GTG AAG GTC GGA GTC AAC G-3'	3	55	445
	GAPDH5.do	5'-AGG AGG CAT TGC TGA TGA T-3'			
<i>ACTB</i> (expression)	Actin.up	5'-ATA TCG CCG CGC TCG TCG TC-3'	3	63	606
	Actin.do	5'- GCT CGG CCG TGG TGG TGA A-3'			
<i>HMGB1</i> (expression)	ORF.up	5'-AAT AAC TAA ACA TGG GCA AAG GA-3'	5	60	659
	ORF.do	5'-TTA TTC ATC ATC ATC ATC TTC TTC TT-3'			
<i>HMGA1</i> (expression)	CodY.up	5'-AGG AAA AGG ACG GCA CTG AGA-3'	7	60	208 and 241
	CodY.do	5'-TCC TCT TCC TCC TTC TCC AGT TT-3'			
<i>HMGA2</i>	1U2.up	5'-AGC ATT CTC ACT CTA ACA CTC GTC-3'	7	60	729
	1R3.do	5'-AGC CAC TGC CAT TTT GTT TTG AG-3'			
<i>HMGN2</i>	G17.up	5'-TGT GGG TGG TTT TCT TCA GT-3'	7	60	524
	G17.do	5'-CTC CCC ATT TCG TAT TAC CA-3'			
<i>HMGB1</i>	HMG1-1147.up	5'-AAG AAG TGT GGG TTT GCT TGG TAA-3'	7	60	994
	HMG1-2117.do	5'-GCA AAG GGA GGA TAA AAC AGT AGG-3'			

Total RNA was isolated from MCF-7 human breast cancer cells using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, not including the optional on-column DNase I digestion. The RNA was quantified using a photometer, and integrity was checked by standard agarose gel electrophoresis revealing the 18S- and 28S-rRNA bands. cDNA first-strand synthesis was performed twice with 2 μg of total MCF-7 RNA using 200 U Maloney murine leukemia virus (MMLV) Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), 1 μM oligo-(dT)₁₇ primer, 0.5 mM each of four dNTPs, 0.01 M 1,4-dithiothreitol (DTT), and first-strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3) in a total volume of 20 μL. The enzymatic reaction was allowed to proceed for 50 min at 37°C and stopped by an incubation for 15 min at 50°C. To eliminate gDNA contamination, 35 μL of the cDNA preparation were digested for 2 h with 10 U DNase I (Roche Diagnostics, Mannheim, Germany) in a buffer containing 10 mM Tris-HCl and 5 mM MgCl₂ (pH 7.4) in a total volume of 60

μL. cDNA was then purified from DNase I by means of the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Finally, the cDNA first strands were eluted in 30 μL elution buffer (Qiagen).

Primer pairs were designed from the cDNA sequences of *GAPDH*, *ACTB*, *HMGB1*, and *HMGA1*, and DNA sequences of *HMGA2*, *HMGN2*, and *HMGB1* using the "Interactive PCR Primer Design" program (available at <http://bibiserv.techfak.uni-bielefeld.de/genefisher/>). RT-PCRs detecting expression of the housekeeping genes *GAPDH* and *ACTB* were performed with 0.5 U *Taq* DNA polymerase (Invitrogen), 200 μM each of four dNTPs, 1.5 mM MgCl₂, 500 nM sense and antisense primer in a final volume of 20 μL. Amplifications were done for 35 cycles as follows: initial denaturation for 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at annealing temperature (see Table 1), and 1 min at 72°C, and a final extension of 10 min at 72°C. RT-PCRs detecting expression of *HMGB1* and *HMGA1* and PCRs of *HMGA2*, *HMGN2*, and *HMGB1* were performed with 0.5 U HotStarTaq™ (Qiagen),

200 μM each of four dNTPs, 1.5 mM MgCl₂, 500 nM sense and antisense primer in a final volume of 20 μL. PCR conditions were as follows: 15 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at annealing temperature (see Table 1), 1 min at 72°C, and a final extension of 10 min at 72°C. The entire PCR was separated in a 1.5% (w/v) agarose gel under standard conditions and stained with ethidium bromide.

In this approach, we synthesized cDNA first strands using RNA isolated from the human breast cancer cell line MCF-7. After first-strand synthesis the cDNA preparation was digested with DNase I to eliminate contaminating gDNA. Finally, we performed RT-PCR and PCR analyses with one aliquot of this cDNA preparation. In all RT-PCRs, MCF-7 cDNA not treated with DNase I served as a positive control. We were able to amplify *GAPDH* and *ACTB* specific fragments of 445 bp and 606 bp using the DNase I-treated MCF-7 cDNA as a template for RT-PCR (Figure 1A), thus revealing the integrity of the corresponding cDNA-sized products after DNase I treatment. The observation that DNase I does not appear to cleave the

DNA-RNA hybrid duplexes that arise in a cDNA first-strand synthesis is consistent with the literature. Sutton et al. (10) showed that DNase I has some structural requirements for cleavage: the enzyme cleaves double-stranded DNA only, and defined distortions in the minor groove significantly affect its cleaving capacity. DNase I shows a preference for the global B-conformation of nucleic acids

but RNA-DNA hybrids are globally in the A conformation (10).

Since *GAPDH* and *ACTB* are considered housekeeping genes, and are thus expected to be expressed at relatively high levels, we also tested the effect of cDNA DNase I digestion on the detection of *HMGB1* and *HMGA1* expression. The *HMGB1* gene is assumed to be strongly expressed in all tissues and

so can be considered a high copy gene (11). In contrast, *HMGA1* expression is extremely weak or not detectable in adult tissues while embryonic and transformed cells show a higher expression or reexpression of the gene (12–15). In MCF-7 cells, the two genes also appear to be expressed to different levels, as assessed by Northern blot (Flohr, unpublished data). Accordingly, *HMGA1* expression is probably more difficult to detect in MCF-7 cells than *HMGB1* expression. Nevertheless, in this study we were able to show by RT-PCR analyses that expression of both *HMG* genes could be detected using the DNase I-treated MCF-7 cDNA as a template for RT-PCRs. These RT-PCRs resulted in a specific 659-bp cDNA fragment of the *HMGB1* gene and two fragments of 208 bp and 241 bp of the *HMGA1* gene representing the two splice variants of the gene (Figure 1B).

We then tested by PCR whether or not the cDNA preparation after DNase I treatment still contains gDNA. To do this, we performed three PCRs detecting genomic sequences of the *HMGA2*, *HMGN2*, and *HMGB1* genes. We could not amplify any genomic PCR product using the cDNA preparation treated with DNase, showing that this cDNA no longer contains gDNA detectable by this assay (Figure 1C). Since these gene-specific PCRs can only detect gDNA that corresponds to the original gene and not to pseudogenes, we cannot exclude the possibility that there is residual contamination with amplifiable gDNA fragments bearing the retropseudogenes. However, since we performed PCRs of genomic fragments of three different genes, it is unlikely that the cDNA preparation still contains residual gDNA.

In contrast, the control PCRs using MCF-7 cDNA not treated with DNase I resulted in a 729-bp fragment of *HMGA2*, a 524-bp fragment of *HMGN2*, and a 994-bp fragment of *HMGB1* (Figure 1C). Furthermore, simply adding the optional on-column DNase I digestion to the standard Qiagen RNA isolation did not eliminate genomic DNA contamination as assessed by an intron-specific PCR of *HMGAZ* (data not shown).

We demonstrate here that a DNase I digestion of cDNA first strands of

Figure 1. RT-PCR analysis of DNase I-treated or untreated cDNA and PCR assessment of genomic DNA contamination. (A) RT-PCRs detecting a 445-bp cDNA fragment of the *GAPDH* gene (lanes 3 and 4) and a 606-bp fragment of the β -actin gene (*ACTB*; lanes 7 and 8) in MCF-7 cDNA with [DNase (+)] and without DNase I treatment [DNase (-)] using the primer pairs GAPDH2.up/GAPDH5.do and Actin.up/Actin.do, respectively. Lane 1: 1 Kb Plus DNA Ladder (Invitrogen, Karlsruhe, Germany); lane 2: water (negative control); lane 3: MCF-7 cDNA / DNase (-); lane 4: MCF-7 cDNA / DNase (+); lane 5: 1 Kb Plus DNA Ladder; lane 6: water (negative control); lane 7: MCF-7 cDNA / DNase (-); and lane 8: MCF-7 cDNA / DNase (+). (B) RT-PCRs detecting a 659-bp cDNA fragment of the *HMGB1* gene (lanes 3 and 4) and two cDNA fragments of 208 and 241 bp of the *HMGA1* gene (lanes 7 and 8) in MCF-7 cDNA with and without DNase I treatment using the primer pairs ORF.up/ORF.do and CodY.up/CodY.do, respectively. Lane 1: 1 Kb Plus DNA Ladder; lane 2: water (negative control); lane 3: MCF-7 cDNA / DNase (-); lane 4: MCF-7 cDNA / DNase (+); lane 5: 1 Kb Plus DNA Ladder; lane 6: water (negative control); lane 7: MCF-7 cDNA / DNase (-); and lane 8: MCF-7 cDNA / DNase (+). (C) PCR detecting a genomic fragment of 729 bp of the *HMGA2* gene (lane 4), of 524 bp of the *HMGN2* gene (lane 9), and of 994 bp of the *HMGB1* gene (lane 14) in MCF-7 cDNA without DNase I treatment but not in MCF-7 cDNA after DNase I treatment using the primer pairs 1U2.up/1R3.do, G17.up/G17.do, and HMGI-1147.up/HMGI-2117.do, respectively. Lane 1: 1 Kb Plus DNA Ladder; lane 2: water (negative control); lane 3: 435 ng genomic DNA (positive control); lane 4: MCF-7 cDNA / DNase (-); lane 5: MCF-7 cDNA / DNase (+); lane 6: 1 Kb Plus DNA Ladder; lane 7: water (negative control); lane 8: 435 ng genomic DNA (positive control); lane 9: MCF-7 cDNA / DNase (-); lane 10: MCF-7 cDNA / DNase (+); lane 11: 1 Kb Plus DNA Ladder; lane 12: water (negative control); lane 13: 435 ng genomic DNA (positive control); lane 14: MCF-7 cDNA / DNase (-); and lane 15: MCF-7 cDNA / DNase (+).

RNeasy-purified RNA yields reverse transcription PCR (RT-PCR) products of the expected size and renders contaminating gDNA undetectable by a PCR assay. Accordingly, this method allows reliable RT-PCR analyses of both genes exhibiting retropseudogenes and genes without introns. There have been reports of primer pairs for *GAPDH* (8) and *ACTB* (16) that were specially designed to exclude amplification of retropseudogenes. However, this method is time-consuming, and appropriate primer pairs have to be designed for every new gene analyzed. By contrast, the procedure described in this report is efficient and can be readily used with any existing primer pairs.

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A rapid assay for the quantitation of γ -glutamyl hydrolase using a fluorogenic peptide as substrate

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Gamma-glutamyl hydrolase (γ -GH) is a lysosomal enzyme that catalyzes the cleavage of natural folylpoly- γ -glutamates, as well as that of the polyglutamates of several antifolate chemotherapeutic drugs (1). Cleavage of polyglutamate residues from antifolates, such as methotrexate (MTX), results in the generation of "free" drug, which is less effectively retained within the cell, thereby reducing its cytotoxicity (2). Increased γ -GH activity has therefore been associated with lower sensitivity to, or resistance against, multiple an-

tifolate drugs (3). These observations and further in vitro (4-6) and in vivo (7-9) studies have suggested that γ -GH activity may be clinically relevant as a possible prognostic marker for the efficacy of chemotherapy in several cancers such as childhood leukemia, for which antifolate therapy is widely used.

Several methods have been developed for the determination of γ -GH activity in cells (10-12). Current protocols measure γ -GH activity by incubating cell extracts in the presence of (radiolabeled) MTX polyglutamates