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Received 16 December 2002; accepted 8 July 2003.

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A/T-rich inverted DNA repeats are destabilized by chaotrope-containing buffer during purification using silica gel membrane technology

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BioTechniques 35:698-702 (October 2003)

The recovery of DNA from agarose gel is a frequently used step in nearly all gene engineering procedures. Various methods have been developed for this purpose, and the use of silica gel or glass matrices seem to be the most efficient ones. Here we describe the difficulties encountered when purifying DNA fragments consisting of inverted repeat sequences.

In our study, we wanted to determine the knock-down phenotype of several novel *Dictyostelium discoideum* genes by using RNA interference (RNAi) (1), which is the method of choice when assaying gene function both in genetically well established and in genetically less well-characterized organisms (2). The RNAi method that we employed consisted of the introduction of double-stranded RNA (dsRNA) molecules (usually several hundred base pairs

long) that are processed into the effector short interfering RNAs (siRNAs; Reference 3), resulting in sequence-specific mRNA degradation (4,5).

Using the sequences of our candidate genes, we constructed an inverted repeat targeting cassette (RNAi cassette) for each gene, which should produce a hairpin RNA upon transcription. Figure 1 depicts the RNAi cassette construction. The identity of the constructed RNAi cassettes was verified by restriction analysis; Table 1 summarizes their properties.

To reclone the prepared RNAi cassettes from the cloning vector into the expression vector, we cut the RNAi cassettes using *Bam*HI, separated them from the vector DNA by electrophoresis on 1% agarose gel, and purified them by gel purification using the QIAquick™ Gel Extraction Kit (Qia-

Figure 1. The RNA interference (RNAi) cassette construction procedure and transcribed RNA structure.

Table 1. Some Properties of the Analyzed RNA Interference (RNAi) Cassettes

RNAi Cassette		Length (bp)	A/T Content (%)
1	DdHAT (SSF192 ^a)	1027	73.22
2	DdHDAC (FC-BE07 ^a)	982	65.88
3	<i>snwA</i> (U43887 ^b) (6)	1412	67.35
4	DdARID (SSD413 ^a)	910	70.99
5	<i>cypE</i> (AF215865) (7)	746	69.98

^aTsukuba cDNA Sequencing Project (<http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html>) (8).

^bGenBank® accession nos. (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA).

gen, Hilden, Germany), according to the manufacturer's instructions. To our surprise, the eluted DNA exerted some unexpected properties. The majority of DNA migrated as a band of approximately two-thirds of the expected size on 1% agarose gel (Figure 2A), and the ligation of the eluted DNA into the expression vector did not yield any transformants carrying the desired constructs (data not shown).

The QIAquick Gel Extraction Kit used for purification employs the chaotropic compound guanidine thiocyanate to dissolve the agarose. Because the *Dictyostelium* genome is known to be A/T rich, the base pairing in our RNAi

cassettes (>65% of A/T pairs; Table 1) could have been less stable and more susceptible to melting in the presence of chaotrope. We hypothesized that such duplex instability eventually lead to the formation of highly favored hairpin structures. The hairpin structures were expected to migrate faster than the original duplex molecules. Indeed, our observations (Figure 2A) were in agreement with this prediction.

We tested whether the chaotropic compound-containing QG buffer (part of the kit) was responsible for the DNA denaturation during the agarose-melting step. We used the RNAi cassette no. 5 for this analysis. For each treatment, a

sample containing approximately 2 µg RNAi cassette DNA was ethanol-precipitated from the restriction reaction mixture using tRNA as a carrier and redissolved in 50 µL EB buffer (part of the kit). The samples were left untreated as a native conformation control (sample 1), heat-denatured at 95°C for 10 min, and slowly cooled at room temperature to serve as a denaturation/renaturation control (sample 2), or subjected to the agarose-melting QG buffer treatment (sample 3). For sample 3, one-tenth of the volume of 10× TAE (400 mM Tris, pH 8.5, 200 mM acetate, 20 mM EDTA) electrophoresis buffer and three volumes of QG buffer were added, and the mixture was incubated at 50°C for 10 min. This treatment recapitulates the procedure recommended by the manufacturer for dissolving a gel slice but does not involve contact of the DNA with the column matrix. The DNA was then ethanol-precipitated from the solution. To show that the effect of the QG buffer cannot be further potentiated by heat denaturation, sample 4 was subjected to the same treatment as in sample 3, followed by heat denaturation as in sample 2. Aliquots (approximately 100 ng of the RNAi cassette no. 5 per lane) were run on 1.5% agarose gel (Figure 2B). This analysis confirmed that the agarose-melting step of the QIAquick Gel Extraction Kit procedure (i.e., the treatment with the QG buffer) was responsible for the mobility shift of the RNAi cassette DNA. The vector shells migrated as expected, and there were no additional bands present.

We reported the problems with the purification of A/T-rich *D. discoideum* RNAi cassettes. We have shown that the agarose-melting buffer (QG buffer) caused destabilization or even denaturation of the short A/T-rich DNA fragments during gel extraction. We propose that this reflects the formation of the hairpin structures that were formed from the kit-destabilized DNA molecules after the denaturing conditions were alleviated. The hairpin structure formation would have rendered the DNA useless for subsequent cloning and would also interfere with the ligation of the few native DNA duplexes left in the solution because it would occupy the vector termini. Agarose electrophoresis followed by gel extraction is a very common method of DNA purification. We

Figure 2. The agarose-melting QG buffer causes denaturation of short A/T-rich DNA fragments. (A) The gel extraction procedure results in altered electrophoretic mobility of the purified DNA. Samples 1–5, containing approximately 2 µg of five different A/T-rich *Dictyostelium* RNA interference (RNAi) cassettes (Table 1), were prepared by the digestion of the respective plasmids with *Bam*HI. One half of each sample was gel-purified using the QIAquick Gel Extraction Kit (+), and the other half was left untreated and used as a control (-). Aliquots corresponding to approximately 200 ng RNAi cassette DNA were adjusted to equal salt concentrations and run on 1% agarose gel. Molecular standards are indicated in base pairs. (B) The QG buffer treatment has the same effect on RNAi cassette DNA as heat denaturation. Four samples that contained equal amounts of the RNAi cassette no. 5 DNA were subjected to the treatments. Lane 3, DNA treated with the QG buffer co-migrated with (lane 2) the heat-denatured control. Lane 4, further heat denaturation of the QG buffer-treated sample did not cause any significant changes. Lane 1 shows an untreated native control. Molecular standards in base pairs are indicated on the left, the positions of the native and denatured RNAi cassette no. 5 DNA are indicated by the arrowhead and the asterisk, respectively. QG, QG buffer treatment; 95°C, heat denaturation. The equal ethidium bromide staining of approximate 750- and 500-bp bands in lanes 1 and 2, respectively, suggests that they have a double-stranded character. M, molecular weight marker (homemade; λ phage DNA digested with *Pst*II).

believe that our findings are of interest to researchers conducting RNAi experiments and/or working with *Plasmodium* and other unicellular pathogens characterized by A/T-rich genomes.

ACKNOWLEDGMENTS

This work was supported by the grant no. 204/02/1512 of the Grant Agency of the Czech Republic (to F.P.).

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Received 9 June 2003; accepted 10 July 2003.

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Complex DNA melting profiles of small PCR products revealed using SYBR® Green I

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BioTechniques 35:702-706 (October 2003)

The fluorescent dye SYBR® Green I (Roche Diagnostics, Mannheim, Germany) binds to the minor groove of the DNA double helix, and its fluorescence is greatly enhanced upon DNA binding. This property is used to monitor DNA amplification during real-time quantitative PCR in the LightCycler® (Roche Diagnostics), a microvolume multicycle fluorimeter with rapid temperature control (1). Products are identified and distinguished by their characteristic melting curves that are dependent on the GC content, length, and sequence of the product (2). However, distinct DNA fragments may have closely overlapping melting profiles and, therefore, a simple melting curve is not sufficient to conclude that a single DNA product is present. Conversely, it is known that longer DNA fragments may have internal melting domains that give rise to complex melting curves (3,4). Smaller DNA products generally melt with a single transition temperature peak (2). Here we show that small DNA fragments may also give rise to more complex melting curves and that the Poland algorithm (5) qualitatively predicts this melting pattern in the majority of cases.

Figure 1 shows two examples of melting curves exhibiting two or more temperature transition peaks and, for comparison, a more typical example, in which a single-peak melting curve is obtained with a single PCR product. The DNA melting patterns predicted by the Poland algorithm (4,5) resemble the observed melting curves, although it is evident in each case that the experimentally observed apparent melting temperature values were significantly higher than those predicted by the algorithm (Figure 1). This is not unexpected because it has been demonstrated that melting temperatures obtained using SYBR Green I fluorescence depend on dye concentration as well as the temperature transition rates (2). Furthermore, for

technical reasons, the solution conditions used by the algorithm could only be approximated to the experimental conditions employed in PCR (for experimental details, see Figure 1 legend). A complex melting curve was also observed when a 188-bp segment of the human β -actin cDNA was amplified (Figure 2). The melting profile was not significantly altered when a slower temperature transition rate (0.05°C/s) was used (data not shown). To gain insight into the origin of this complex melting behavior, we performed additional experiments. The presence of a single 188-bp DNA product was confirmed by agarose gel electrophoresis. To exclude the possibility of co-migrating unrelated DNA, the reaction product was purified and subjected to DNA sequencing, which confirmed the 188-bp sequence derived from human β -actin. It is of note that this DNA product (5'-TTGCGTTACACCCTTTCTTGACA-AAACCTAACTTGCGCAGAAAA-CAAGATGAGATTGGCATGGCTTT-ATTTGTTTTTTTTTGTGTTTGTGTTTGTGTTTTTTTTTTTTTTTGGC-TTACTAGGATTTAAAAACTG-GAACGGTGAAGGTGACAG-CAGTCGGTTGGAGCGAGCA-TCCCCAAAGTTCACAAT-3') contains a centrally located 44-bp A/T-rich region. To determine which subregion contributed to the complexity of the observed melting curve, we cloned the β -actin PCR product and used the resultant plasmid to amplify segments from within the 188-bp insert, as shown schematically in Figure 2A. Agarose gel electrophoresis of the resultant PCR products demonstrated the presence of a single band in each instance (Figure 2D). The predicted and observed melting curves for each of these products are also shown (Figure 2, B and C). As expected, the 67- and 61-bp products, corresponding to the regions 5' and 3' to the A/T-rich segment (primer pairs 1F-2R and 3F-1R,