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ARTICLE

Determination of Optimal Conditions for Analysis of p53 Status in Leukemic Cells Using Functional Analysis of Separated Alleles in Yeast

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Tumor suppressor p53 is transcription factor that participates in control of many cellular functions. Somatic mutations of the p53 gene are frequently detected in human cancers. Several methods can be used for identification of p53 mutations, including FASAY – functional analysis of separated alleles in yeast. FASAY distinguishes yeast colonies expressing functional p53 protein from colonies producing a dysfunctional p53 protein simply on the basis of color. The validity of the method depends on a low background level. There are several sources of background as PCR-induced point mutations, low quality of RNA and alternative splicing of intron 9 affecting the p53 carboxy-terminus. In the present work we show that FASAY can be successfully used for analysis of mRNA isolated from blood samples that were collected and stored for 24 hours at 0°C without undesired increase of background. We also measured fidelity of several commonly used DNA polymerases and determined the most suitable kinds of Pfu DNA polymerases for FASAY. Reaction conditions described in this report allow routine analysis of p53 status in leukemic cells using FASAY. (Pathology Oncology Research Vol 8, No 4, 245–251)

Keywords: tumor suppressor p53, FASAY, DNA polymerase fidelity, mRNA stability

Introduction

Tumor suppressor p53 is a transcription factor that participates in control of many cellular functions. Somatic mutations of the p53 gene are frequently detected in human cancers and the p53 status is increasingly regarded as a predictive marker in several types of cancer. Several methods have been developed for identification of p53 mutations in clinical material.[18] Among them, FASAY – functional analysis of separated alleles in yeast[7,11] – has been recently proved to be highly sensitive and reliable.[5,13,14]

This ingenious test distinguishes yeast colonies expressing functional p53 protein encoded by normal p53 cDNA sequence from colonies producing a dysfunctional p53 protein encoded by mutant cDNA simply on the basis of color[7] (*Figure 1*). The central part of the p53 gene derived from mRNA of tumor cells by RT-PCR is introduced into Ade- yeast strain carrying a reporter with a p53-binding site ribosomal gene cluster (RGC) upstream of the ADE2 gene. Yeast cells grown in agar plates containing a low level of adenine form either bigger white colonies if the reporter is activated by functional p53 or smaller red colonies if the reporter is not activated. The sensitivity of FASAY is limited by background of the method. Cells or tissue containing only wild-type p53 do not score 100% of white yeast colonies when analyzed by FASAY, but provide 5–10% of red colonies instead. Main sources of this background are PCR-induced point mutations,[6] low quality of RNA[20] and alternative splicing of intron 9 affecting the p53 carboxy-terminus.[8]

FASAY was originally developed to detect germ-line and somatic p53 mutations in clinical material and it was successfully used for this purpose many times.[3] But FASAY proved to be a method of much wider use. For example, the method was used for the isolation of experimentally induced p53 mutants and for studies of mutational spectra of p53.[10] FASAY was applied to detect alternatively spliced p53 mRNA,[8] as a rapid assay of DNA polymerase fidelity[6] and for analysis of partially inactivating p53 mutations.[4,9] Previously, we used FASAY for p53 status analysis in breast carcinoma.[16,17] In our next project, we intended to use the method for analysis of p53 status in

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leukocytes of patients with acute myeloid leukemia. We could not exclude a time delay in blood sample processing. Therefore, we wished to assess the sensitivity of the FASAY to RNA degradation. We found that quality of mRNA reflected by FASAY was not significantly changed during the first 24 hours of storing at 0°C after blood sample collection in case when efficient amplification of p53 cDNA could be achieved. We also measured the fidelity of some commonly used DNA polymerases by FASAY. We have identified four excellent enzymes from the set of tested DNA polymerases that scored the highest fidelity: Pfu DNA polymerases (Stratagene, Promega and Fermentas) and Pyrobest (Takara Shuzo). We recommend these enzymes for routine usage in FASAY.

Material and Methods

Sample processing

Leukocytes were isolated from peripheral blood using Erythrocyte Lysis Buffer (Quigen Inc.) according to the instructions of the manufacturer. Cells (cultured cell lines, leukocytes, tumor tissue) were immediately homogenized in RLT lysis buffer (Quigen Inc.) and then either kept at –70°C or immediately used for RNA preparation.

FASAY

FASAY was performed using the protocol described by Flaman et al.[7] with several modifications. Shortly, total cellular RNA was purified from cells using RNeasy Mini Kit (Quigen Inc.). cDNA was synthesized by SuperScript II (Life Technologies Inc.) using oligo$(dT)_{12}$ as a primer. PCR was performed as described [7] using primers P3 (5´-CCT-TGC-CGT-CCC-AAG-CAA-TGG-ATG-AT-3´), complemetary to nucleotides 101-126 of the p53 gene, and P4 (5´-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCT-GGA-GT-3´) complementary to nucleotides 1094-1122 of the p53 gene, and Pfu DNA Polymerase (Stratagene). Ethidium bromide stained PCR product was analyzed by electrophoresis in 1,2% agarose gel. Yeast cells were cotransformed with the PCR product, linearized vector pSS16, and Salmon Sperm DNA carrier (Life Technologies Inc.) by lithium acetate procedure, as described by Ishioka et al.[11] Transformed yeast cells were plated on minimal medium containing a low amount of adenine (5 μg/ml) and lacking leucine and incubated at 35°C for 2-3 days and then at RT for 2-3 days. The percentage of white and red colonies was then determined; 100–200 colonies per case were usually assessed.

In the experiments testing different DNA polymerases, FASAY was performed according to the same protocol, with the exception of PCR that was set up exactly according to the manufacturer´s protocols.

Statistical analysis

A relationship between the percentage of red colonies and the content of mutant p53 DNA was analyzed by regression analysis. Analysis of time delay in sample processing was performed using the Wilcoxon matched pairs test, the Friedman test and the Mann–Whitney test. Differences were considered significant at $P<0,05$.

The statistical analyses were performed using statistical software Statistica (StatSoft, Inc.).

Results

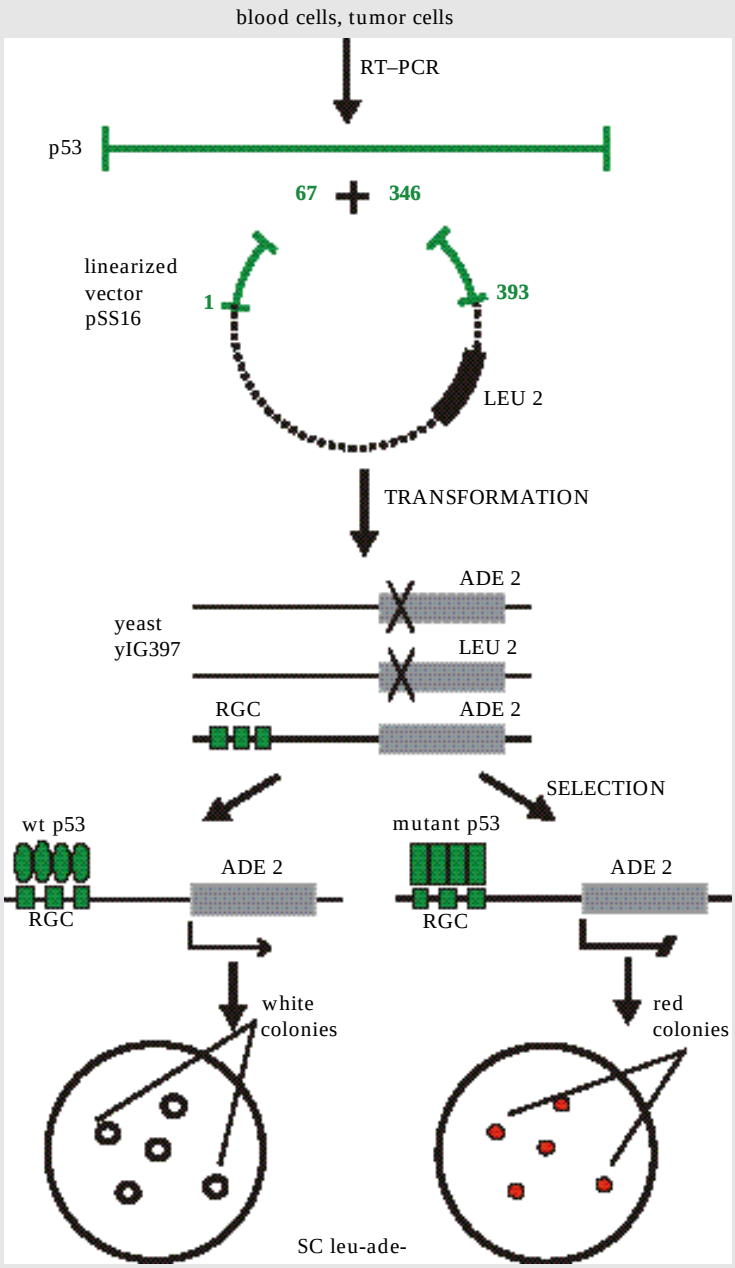
FASAY scores correctly p53 status of analyzed samples

In order to check fidelity of the method, we used plasmid pC53-SN3 carrying wild-type p53 cDNA[1] and plasmid cmvHup53Trp248 carrying mutant p53 cDNA (kindly provided by Carol Midgley), mixed them at serial ratios and used the mixtures as templates for PCR. PCR products were then used for transformation of yeast together with gapped pSS16 plasmid, and the percentage of red colonies was scored. As shown in *Figure 2,* a percentage of red colonies was directly proportional to the content of mutant p53 DNA in the sample (Pearson r, P 0,001). This documents that FASAY is a reliable method reflecting precisely the level of mutant p53 templates in tested material.

Second, we used leukemia cell lines with known p53 status for analysis by FASAY. Chronic myeloid leukemia cell line Bv173, which contains wild type (wt) alleles of p53 gene[2,15] scored 2.9% of red colonies by FASAY. Promyelocytic leukemia cell line NB4, which contains mutant (mut) p53[15] scored 100% of red colonies. Myeloid cell line ML-1 is heterozygous (het) for p53 gene providing 47.2% of white and 52.8% of red colonies. These results confirm that FASAY correctly assesses p53 status in tested cells.

Figure 1. Scheme of FASAY[7] (A). RNA isolated from blood cells is reverse transcribed into cDNA and the central part of the p53 gene is amplified by PCR. Yeast cells yIG397 are then cotransformed with PCR product and gapped expression vector pSS16 carrying the 5´and 3´ termini of p53 gene and LEU2 selection marker. PCR product is then cloned into pSS16 by homologous recombination in vivo (gap repair), resulting in constitutive expression of p53 protein. Host yeast strain yIG397 is leu$^-$ ade$^-$ and contains an integrated plasmid with ADE2 open reading frame under control of minimal promoter and p53 binding site RGC. Cells transformed with plasmid encoding wild-type p53 express ADE2 and form white colonies of regular size (B) on selective medium, which lacks leucin and contains only minimal amounts of adenine sufficient for growth of Ade$^-$ cells. Cells containing mutant p53 do not express ADE2 and form small red colonies (C) because of accumulation of intermediate in adenine metabolism.[19]

A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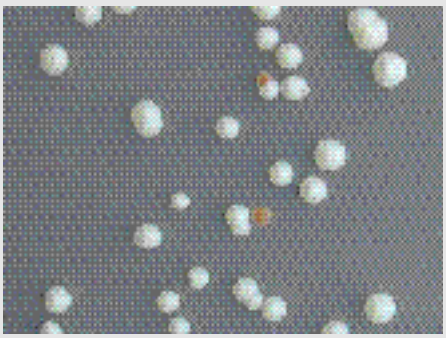

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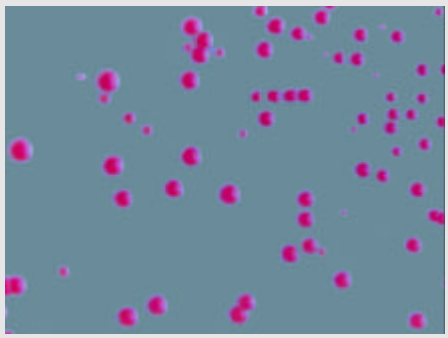

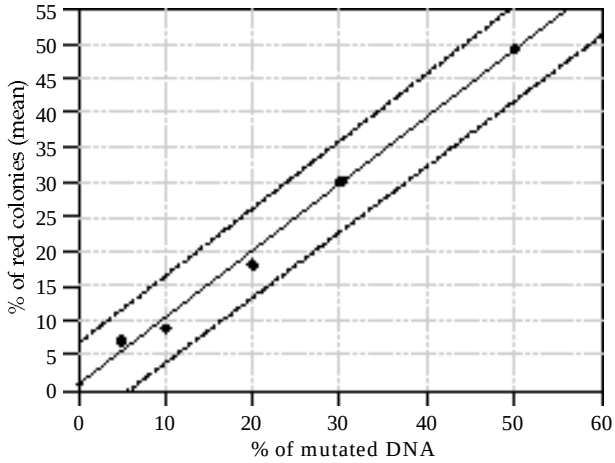

Figure 2. Relationship between the content of mutant p53 cDNA in sample and the percentage of red colonies scored by FASAY. Positive correlation was evaluated by Pearson correlation coeficient, P< 0,001.

The role of DNA polymerase fidelity for FASAY

As mentioned above, PCR-induced point mutations are one of the main sources of background of red colonies detected by FASAY.[6] Thus, validity of FASAY is dependent on the fidelity of DNA polymerase used for amplification of p53 gene. We tested several DNA polymerases to find the best enzyme for FASAY. First, we tested enzymes previously successfully used by others or described as high fidelity systems by manufacturers. Later, we extended our studies of DNA polymerases because the information about enzyme fidelity provided by FASAY could be important to the scientific community for various other purposes (*Table 1*).

For testing of DNA polymerase fidelity, we do not usually perform complete FASAY beginning with RNA isolation from tested material. Instead, we directly use plasmid pC53-SN3 coding for wild-type p53[1] as a template for PCR. This arrangement significantly reduces the other sources of undesired background, especially low quality of mRNA. On the other hand, this arrangement is not usable for one-step RT-PCR systems, such as the Titan™ One Tube RT-PCR System (Roche). Therefore, the complete FASAY was also performed for all tested DNA polymerases with only one exception (DyNAzyme™ EXT – Finnzymes).

We found four enzymes from the set of tested DNA polymerases that scored the highest fidelity providing up to 3.5% of red colonies with cDNA as template and up to 10% of red colonies with RNA as template: Pfu DNA polymerases (Stratagene, Promega, Fermentas) and Pyrobest (Takara Shuzo). We recommend these four enzymes for routine usage in FASAY. Also, the Titan™ One Tube RT-PCR System (Roche) scored in the complete version of FASAY under 10% cut-off for number of red colonies and it is thus suitable for the assay.

The role of p53 mRNA stability for FASAY

It has been shown that high-quality p53 RNA is required for FASAY.[7,20] We tested how delay in sample processing (for example during sample transport) influences the quality of p53 mRNA and results of FASAY. We collected peripheral blood of eleven healthy donors and let it to stay either at room temperature or at 0°C for 0h, 3h, 6h, 12h and 24h. Aliquots of the blood were used for preparation of leukocytes and their homogenization in RLT in the time intervals. The lysates were either kept in –70°C or immediately used for RNA preparation. Then, FASAY was performed and white and red colonies were counted. Surprisingly, the time delay in sample processing resulted in only a slight increase of percentage of red colonies *(Figure 3).* For samples kept at 0°C, the increase of background was not statistically significant (based on

Table 1. **Fidelity of DNA polymerases: percentage of red colonies scored by FASAY using either plasmid pC53-SN3 coding for wild-type p53 (cDNA) or RNA isolated from cells containing wild-type p53 genes (RNA).**

DNA polymerase	*Proofreading activity*	*% of red colonies*	
		template	
		cDNA	*RNA*
Pfu (Stratagene)	+	1,5–3,5	2,5–8
Pfu (Promega)	+	1,5–3,5	2,5–8
Pfu (Fermentas)	+	2,2	5,1
Platinum Pfx (Life Technologies Inc.)	+	7,6	11,6
Deep $Vent_R$ (New England BioLabs)	+	6,4	9,1
Pyrobest (Takara Shuzo)	+	1,4	3,3
AccuTaq LA (Sigma)	+/2	16,2	25,6
ExpeRT-PCR™ (Hybaid)	+/2	10,8	14,9
Titan™ One Tube RT-PCR (Roche)	+/2	–	7
DyNAzyme™ EXT (Finnzymes)	+/2	8,7	ND
Taq (Life Technologies Inc.)	–	13,5	20–25
Taq (Sigma)	–	12,5	20–25

The enzymes declared as possessing integral 3´→5´ proofreading exonuclease activity are indicated as "+". DNA polymerases, which are mixtures of two enzymes – one with highly processive activity and the other with proofreading activity are indicated as "+/2". DNA polymerases with no declared proofreading activity are indicated as "–". The numbers indicate either a range from minimal to maximal values of multiple experiments (Stratagene- and Promega Pfu-polymerases, Sigma- and Life Technologies Taq-polymerases) or the average of two independent experiments.

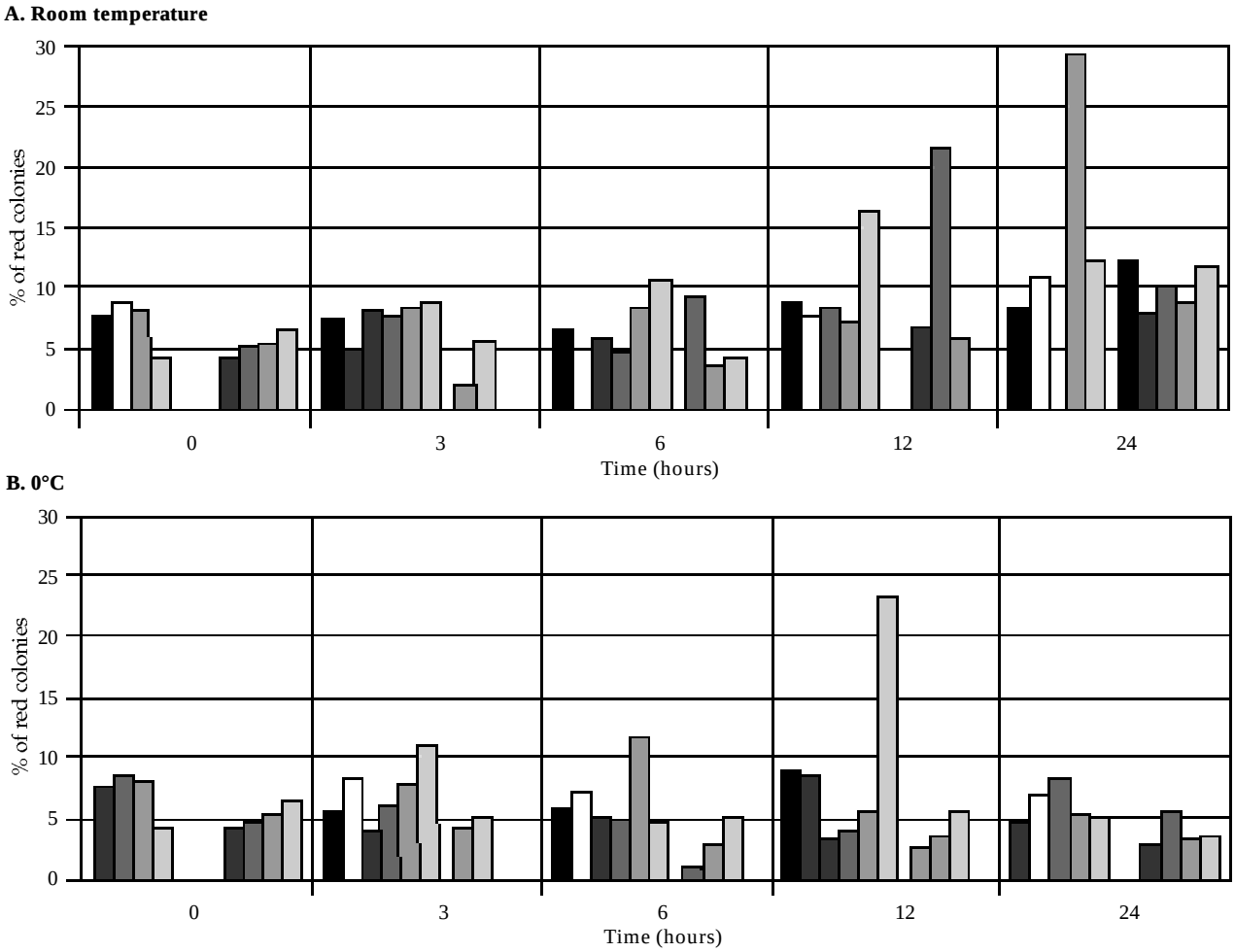

Figure 3. Relationship between time delay of blood sample processing and background level. Blood samples of eleven healthy donors were kept for indicated time either at room temperature (A) or at 0°C (B). *Then FASAY was performed and percentage of red colonies was counted. The usual cut-off level 10% is indicated.*

Wilcoxon matched pairs test and Friedman test). For samples kept at room temperature, the statistically significant increase of background was reached after 24 hours (based on Wilcoxon matched pairs test, $P<0.05$), the increase after 12 hours was near significant. Tested altogether by Friedman test, the increase in red colony percentage in samples kept at room temperature was near significant ($P=0.076$). When we compared background level in samples kept either at 0°C or at room temperature, the statistically significant difference (based on Man–Whitney test; $P<0.05$) was found for the 24 hours time interval confirming higher mRNA stability at 0°C.

Discussion

FASAY scores correctly p53 status of analyzed samples

We showed that the FASAY correctly scored the p53 status in tested cells and reflected correctly the level of mutant p53 templates in analyzed sample, too. The results presented in *Figure 2* are in average 2–4% lower than expected. For example, we expected that a sample with 50% of mutant DNA would provide 51.5–53.5% of red colonies, because we expect a background up to 3.5% of red colonies. The discrepancy certainly reflects the inaccuracy of a method used to adjust DNA concentration, which was done by using of spectrophotometer, but does not impair the close correlation between the content of mutant p53 DNA in the sample and percentage of red colonies.

Fidelity of DNA polymerases scored by FASAY

Some DNA polymerases possess besides the $5'\rightarrow3'$ polymerization activity also $3'\rightarrow5'$ proof-reading exonuclease activity. The proof-reading activity allows them to remove mismatches in case of insertion of a wrong nucleotide by DNA polymerase that increases their fidelity. We tested the fidelity of several DNA poly-

merases by FASAY. We found that only DNA polymerases with proofreading activity are precise enough to be routinely used in FASAY for clinical material analysis. From our results we can clearly recommend Pfu polymerases from Stratagene, Promega and Fermentas or Pyrobest from Takara Shuzo. Also the Titan™ One Tube RT-PCR System (Roche) is an enzyme system with high fidelity, but this is not usable for two-step RT-PCR. We ourselves use Pfu polymerase (Stratagene) or Pfu polymerase (Promega) in our projects with satisfactory results. We know that also other DNA polymerases are used for that purpose, for example Pfu polymerase (Roche),[13] Pfu polymerase (Eppendorf)[10] and others.

Other enzymes not tested in the present work may also be useful for FASAY. In our tests, PCR reactions for individual DNA polymerases were set up exactly according to the manufacturer´s protocols without any optimization. Thus, we cannot exclude that better results (in term of enzyme fidelity) could be reached with these enzymes by changing some parameters of the PCR reaction such as dNTP concentration or Mg^2 concentration.[6] Similarly, we cannot rule out the possibility that lower number of PCR cycles would decrease the background of FASAY. In this study we always used 35 PCR cycles. These conditions are commonly used for FASAY by other authors as well.

We also like to point out that by using FASAY we tested fidelity as the only parameter of the DNA polymerases. There are also other parameters which can differ significantly among the enzymes and which could make the DNA polymerases enzymes of choice for different purposes. For example, some DNA polymerases are highly processive and efficient and thus provide significantly higher yield. The processivity and fidelity is balanced in other DNA polymerases thus allowing them to be used for successful amplification of very long templates. Some enzymes are modified in the way allowing “hot start” PCR and so on.

Degradation of p53 mRNA

There are three main sources of background in FASAY: PCR-induced point mutations, an alternative splicing of intron 9 and degradation of RNA. There have been several recommendations set up in order to reach the lowest background. One of them was an immediate stabilization of mRNA after sample collection. To assess the reasonable time limit for blood sample processing we measured correlation between time delay and background level. We expected to get some increase of background in the course of time. Surprisingly, the background did not change significantly. This suggests that the quality of mRNA determined by FASAY is not significantly decreased within the interval 0–24 h at 0°C. On the other hand, FASAY does not reflect the quantity of mRNA, because to some point FASAY is not affected by mRNA level. For optimal results, transformation of yeast was always performed with “the standard” amount of PCR product estimated from agarose gel electrophoresis. The delay in blood processing influences the total amount of RNA isolated from leukocytes and the overall amount of PCR product. The increasing time delay complicates getting sufficient RNA and thus a sufficient amount of PCR product. This problem clearly results from progressive RNA degradation. However, once we had the “standard” level of PCR product, background of FASAY was usually under the level of 10%.

This result suggests that RNA degradation results in disqualifying mRNA as a template for RT-PCR rather than in damage causing increase of background of red colonies.

On the other hand, we know from our long-term experience that once the efficiency of cDNA amplification is low, the background of FASAY increases. It has been shown that many of the “background p53 mutants” contain intragenic deletions, probably as a result of PCR splicing of fragmented cDNA.[20] It is possible that the suggested PCR splicing of fragmented cDNA happens predominantly in samples where the level of templates of good quality is rather low and degraded mRNA “has a chance” to be used as a template. In the samples where the level of high-quality templates is high, “the good templates” are probably used preferentially and degraded mRNA templates thus cannot significantly affect FASAY. Although we show that it is possible to keep background of the FASAY on low level even with progressively degraded RNA, the reproducibility of the method decreases. It is illustrated by randomly increased background of some samples processed after 12 or 24 hours of delay.

In conclusion, FASAY is a method, which has been used for several different purposes and has produced many interesting results. Originally, it was developed as method for routine detection of germ-line and somatic p53 mutations. Several rules were earlier described, which should be followed for successful usage of the method. For example, samples should be processed immediately, p53-specific primers should be used for reverse transcription rather than random hexamers, mRNA should be isolated rather than total RNA and high fidelity proofreading DNA polymerase should be used for PCR.[20] We confirm in the present work the enormous sensitivity of FASAY and the importance of usage of a high fidelity DNA polymerase. On the other hand, we demonstrate that some time delay in sample processing is not disqualifying for FASAY, once the efficient amplification of p53 cDNA has been achieved.

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