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Rapid Quantification of Telomerase Activity Employing an Improved Real-time Telomeric Repeat Amplification Protocol in Clinical Tissue Samples Eliminates Interference by PCR Inhibitors

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Abstract

Telomerase, a ribonucleoprotein with reverse transcriptase activity, enables human cells to maintain chromosomal stability and to proliferate without limits. Various studies demonstrated telomerase activation in human cancer, including hepatocellular carcinoma. Therefore, quantification of telomerase activity has been proposed as diagnostic and prognostic tool. In this study, we optimized a 1-step real-time quantitative telomeric repeat amplification protocol for the robust and rapid quantification of telomerase activity in clinical tissue samples. To ensure undisturbed PCR kinetics even in samples with high telomerase activity, we initially determined optimal sample dilution for our assay. Next, we assessed highly diluted samples and did not observe relevant interference by tissue inhibitors of PCR, which constitute a major problem analyzing clinical tissue samples with end-point assays. To test our real-time assay, we evaluated human liver samples and detected increased telomerase activity in malignant liver lesions, whereas benign liver tissue displayed only minimal telomerase activity. In conclusion, our optimized assay is suitable to quantify telomerase activity in clinical tissue samples without interference by PCR inhibitors. The assay may be employed to detect telomerase activity during carcinogenesis and to monitor telomerase activity during cancer progression and treatment.

Keywords: Telomerase activity; Real-time quantitative TRAP; PCR-inhibitors; Hepatocellular carcinoma

Abbreviations: C_t: Threshold cycle value; HCC: Hepatocellular carcinoma; IC: Internal control; NTC: No template control; RTA: Relative telomerase activity; TRAP: Telomeric repeat amplification protocol; tRQ-TRAP: Tissue real-time quantitative telomeric repeat amplification protocol

Introduction

Telomerase, a ribonucleoprotein with reverse transcriptase activity, plays a pivotal role in maintaining telomere length and chromosomal stability in proliferating mammalian cells. In cells without telomerase activity, replication-associated telomere attrition limits the replicative lifespan [1]. Therefore, telomere maintenance, mostly through telomerase activation, has been described as prerequisite for cancer [2]. As activation of telomerase is considered to be a crucial step in carcinogenesis [3,4], telomerase has been proposed as tumor marker for various malignancies [5], including hepatocellular carcinoma (HCC) and liver metastases [6]. Thus, a highly sensitive and reliable test to precisely quantify telomerase activity in biopsy samples would be useful to evaluate suspicious lesions. Telomerase activity can be detected by the sensitive telomeric repeat amplification protocol (TRAP) introduced by Kim et al. [7]. However, this technique is time-consuming and susceptible to carry-over contamination because it requires post-PCR polyacrylamide gel analysis and cannot be performed as closed-tube assay. Even more importantly, reliable quantification of telomerase activity by conventional TRAP assays requires the use of radioactive nucleotides restraining its application in high-throughput clinical analyses. To circumvent the need for post-PCR analysis and to obtain quantitative data without the use of radioactivity, the standard TRAP assay was modified by various groups. For example, Uehara et al. [8] employed energy transfer primers that emit fluorescence upon incorporation into PCR products, which allows telomerase detection in a closed-tube system without post-PCR analysis. Furthermore, to measure telomerase activity in cultured cells some investigators have developed real-time TRAP assays using fluorescent dyes or probes to monitor product generation during amplification [9,10]. To further promote clinical application of the TRAP assay, Jakupciak et al. [11] reported an automated high-throughput system for the measurement of telomerase activity in cultured cells and body fluids.

In this study, we measured telomerase activity in clinical tissue samples using a procedure based on the real-time quantitative TRAP introduced by our group [12]. The procedure described here constitutes an improvement of our original protocol and requires less hands-on time than standard TRAP assays while generating equally reliable results. The real-time quantitative TRAP was initially developed to monitor telomerase activity in cultured cells. Potential problems analyzing clinical tissue samples instead of cultured cells are at hand: (i) there is only a small amount of tissue available, (ii) the quality of the tissue samples is often not eligible for telomerase quantification [13],

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and (iii) there is an increased risk of false negative results due to tissue inhibitors against Taq polymerase [14]. In fact, presence of inhibitory activity in clinical tissue samples often necessitates sample dilution down to or below the lower detection limit of TRAP [15]. In this study, we addressed these issues, optimized the real-time quantitative TRAP to assess clinical tissue samples, and evaluated a set of human liver samples representing different benign and malignant lesions. The presented results support translation of our tissue real-time quantitative TRAP (tRQ-TRAP) into the clinical diagnostic setting.

Materials and Methods

Human liver samples

Snap-frozen liver samples were obtained from the human tissue repository at our institution (Department of Hepatobiliary Surgery and Visceral Transplantation, University Medical Center Hamburg-Eppendorf). Tissue collection was under approval by the local ethics committee. Histological results were provided by the local pathology service (Department of Pathology, University Medical Center Hamburg-Eppendorf). HCC samples were graded as well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) according to the Edmondson-Steiner grading system [16]. To evaluate the optimized tRQ-TRAP, a set of 15 human liver samples was investigated: 8 HCC, 2 liver metastases from colorectal carcinomas, 1 liver adenoma, and 4 normal liver samples.

Evaluation of RNA integrity

Telomerase is an RNase-sensitive ribonucleoprotein. Thus, RNA integrity is an important marker for tissue quality when analyzing telomerase activity [13]. To ensure sufficient RNA quality, total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNA quality was assessed by denaturing agarose gel electrophoresis. Only samples with sharp 18S and 28S rRNA bands with a ratio of approximately 2:1 and without degradation were considered for telomerase quantification.

Sample extraction and tRQ-TRAP

TRAP extracts were prepared from approximately 20 mg tissue with CHAPS lysis buffer as described [7]. Total protein content was determined with the Compat-Able Protein Assay (Perbio Science, Bonn, Germany). Protein concentration was employed to guide sample dilution and standardization. For the tRQ-TRAP, we used the ABI Prism 7900 thermal cycler (Applied Biosystems, Foster City, CA). The reaction mixture consisted of 20 µl SYBR-Green I Universal PCR Master Mix (Applied Biosystems), 2 µl primer mix containing 80 ng/µl of the telomeric primer TS (5'-AATCCGTCGAGCAGAGTT-3') and 40 ng/µl of the anchored reverse amplification primer ACX (5'-GCGCGG $[CTTACC]_3$CTAACC-3'), and 13 µl PCR-grade water for a total reaction volume of 40 µl (5 µl sample volume with 20 ng protein in the optimized protocol). TS and ACX primers were originally developed by Kim and Wu [17]. The PCR protocol started with an incubation step of 20 minutes at 25°C to allow elongation of TS primers by telomerase, followed by Taq polymerase activation for 10 minutes at 95°C (hot-start PCR), and 40 amplification cycles with denaturation at 95°C for 30 seconds and elongation for 1 minute at 60°C (two-step PCR). Every run included extracts from serially diluted telomerase-positive 293T cells (equivalent to 1000, 500, 100, 50, and 10 cells, respectively) as standards, heat- and RNase-inactivated samples as negative controls, and no template controls (NTC) to monitor primer-dimer formation. Following cycling, a standard curve was derived from semi-log amplification plots using the crossing cycle value (C_t) of each 293T amplification plot with a horizontal threshold of significant amplification set above NTC signals. Finally, telomerase activity was expressed as relative telomerase activity (RTA) compared to 293T standards, i.e. the number of 293T cells required for an equivalent telomerase activity.

Telomerase inactivation

To generate negative controls, telomerase was inactivated in diluted samples (1000 ng protein per 50 µl) by heat-denaturation at 70°C for 20 minutes. Alternatively, the essential RNA component of the telomerase complex was digested at 37°C for 20 minutes with DNase-free ribonuclease A (Sigma, Taufkirchen, Germany) at a final concentration of 50 µg per 50 µl.

Commercial TRAP assays

As additional assays, we used two commercially available kits, the TeloTAGGG Telomerase PCR ELISA^{PLUS} (Roche Diagnostics, Mannheim, Germany) with 1000 ng protein per 50 µl reaction volume and an initial incubation time of 20 minutes, and the TRAPeze (Serologicals Corporation, Norcross, GA) with 500 ng protein and 30 cycles per reaction.

Statistical analysis

C_t values and RTA of repeat measurements are expressed as means ± SEM. Correlation between diluted samples and C_t values or RTA was determined using a rank order test and the Spearman correlation coefficient (R). Because of the small number of samples, comparison between groups (HCC versus normal liver tissue) was performed with the non-parametric Mann-Whitney U test. $P < 0.05$ was considered as statistically significant.

Results

Relevance of tissue amount for TRAP extraction

To test the relevance of different tissue amounts for TRAP extraction on our system, we prepared extracts from varying amounts of tissue (1 mg to 20 mg) and analyzed 20 ng protein per reaction. We observed slightly lower RTA values in TRAP extracts prepared from larger tissue amounts. However, there was no significant correlation between the amounts of tissue used for TRAP extraction and the corresponding RTA values. Moreover, considering the standard error of our measurements, RTA values were overlapping without apparent trend; sample 130 (HCC, G2): 1 mg tissue 163 ± 28 (mean RTA ± SEM, $n = 3$), 5 mg tissue 153 ± 26, 10 mg tissue 145 ± 40, 20 mg tissue 144 ± 11 (rank order correlation test, $P = 0.0833$). TRAP extracts generated from as little as 1 mg tissue yield reliable telomerase activity data.

Influence of PCR inhibitors

To address the problem that tissue extracts might contain inhibitors of Taq polymerase as reported by others, [14,15] we analyzed our samples with the TeloTAGGG Telomerase PCR ELISA^{PLUS} and TRAPeze. Both commercially available assays include an internal control (IC) to monitor PCR inhibition and to rule out false-negative results. In the TeloTAGGG Telomerase PCR ELISA^{PLUS} (1000 ng protein per 50 µl), absence of IC amplification and lack of detectable telomerase activity was observed in five samples marked with an asterisk in Figure 2. Four of these samples (sample 8, 23, 2569, 148 I) did not show an apparent ladder of products with 6 base increments in the TRAPeze (500 ng protein per 50 µl), despite detectable IC amplification. The typical

product ladders are shown in Figure 2. In contrast, these samples had readily detectable telomerase activity evaluating higher diluted samples (20 ng protein per 40 µl) with the tRQ-TRAP.

To further investigate interference of PCR inhibitors with the tRQ-TRAP, samples were inactivated both by heat or RNAse-treatment and serially diluted from 1000 to 1 ng protein per reaction. A telomerase-positive standard (equivalent to 100 293T cells) was spiked into each reaction to create "mixed" samples with known telomerase activity (inhibitor control sample). All "mixed" samples and controls amplified as expected for the telomerase positive standard alone. We could not detect any correlation between the amount of inactivated sample and the C_t values generated. As demonstrated in Figure 3, C_t values were comparable to the 100 293T cell-standard without inactivated TRAP extract.

Evaluation of human liver samples with the tRQ-TRAP

Mean RTA values of repeat measurements for all groups of samples are summarized in Figure 4. As reported by others, [4,19] HCC samples showed significantly higher telomerase activities compared to normal liver tissue (G2 and G3 HCC, n = 8, median RTA 51.8 versus normal liver, n = 4, median RTA 8.0; Mann-Whitney U test, P = 0.018) and liver adenoma. All G3 samples showed markedly elevated RTA levels compared to normal liver tissue, whereas only 3 out of 5 G2 samples had elevated RTA levels (above mean RTA + 2 SEM of normal liver samples).

Discussion

TRQ-TRAP appears to be a sensitive method to quantify RTA in liver tissue samples. The measurements are not biased by the amount of starting material used to prepare TRAP extracts and even extracts from as little as 1 mg of starting tissue showed reliable results.

Reliable quantification of telomerase activity without interference by tissue inhibitors of Taq polymerase is very important for high-throughput clinical applications. The data demonstrate that PCR inhibitors interfering with performance of the TeloTAGGG Telomerase PCR ELISA^{PLUS} have less relevance using more dilute samples and a different PCR master mix without EGTA in the tRQ-TRAP. We have not detected any interference of PCR-inhibitors evaluating highly diluted TRAP extracts of liver samples. However, because we cannot rule out interference by tissue inhibitors in other tissue samples, for example colon, which has been reported to contain higher inhibitory activity [18], an inhibitor-control sample should be incorporated into the tRQ-TRAP as suggested in Figure 1.

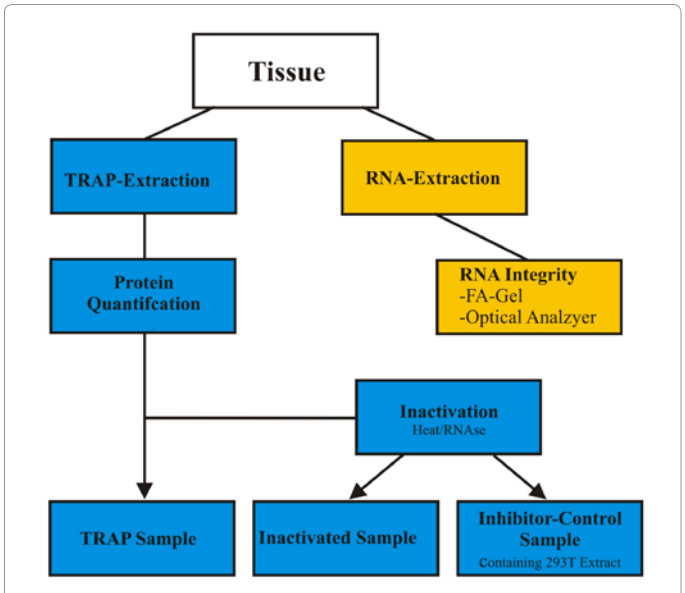

Figure 1: The tRQ-TRAP includes RNA extraction to verify sufficient tissue quality by formaldehyde agarose (FA) gel analysis or an optical analyzer as well as inactivated and inhibitor-control samples to rule out false-positive and false-negative results. All steps are described in detail under Materials and Methods.

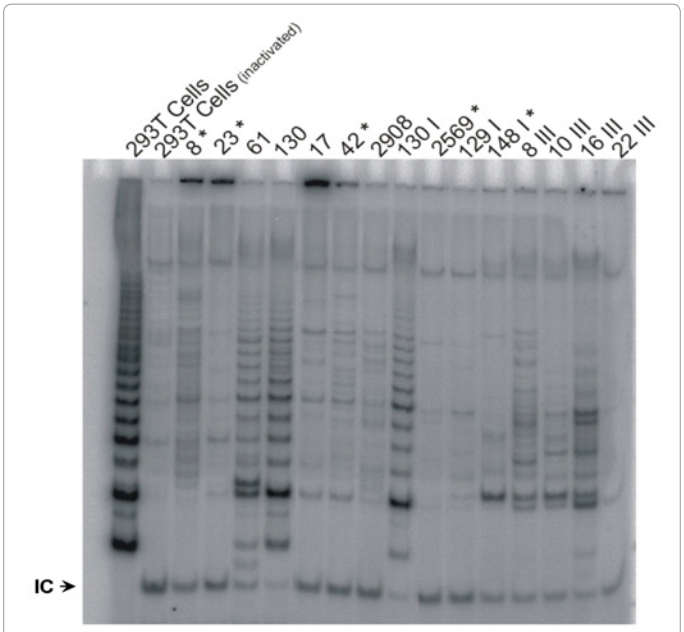

Figure 2: Post-PCR polyacrylamide gel analysis using the TRAPeze (Serologicals Corporation) confirms the typical ladder of products and amplification of the internal control (IC). 293T cells were evaluated as positive control and inactivated 293T samples served as negative control. Samples marked with an asterisk displayed absence of IC amplification in the TeloTAGGG PCR ELISA^{PLUS} (Roche Diagnostics).

A relationship between tumor grading and telomerase activity was reported by other groups [20]. The levels of RTA were significantly higher in HCC samples compared to normal liver tissue. Moreover, a tendency for higher RTA values was observed in G3 compared to G2 tumors. Overall, 75% of HCC samples displayed an increase in telomerase activity. Other groups reported increased telomerase activity in 68 to 85% of HCC samples [14,19,20].

There are several limitations to our results. First, there was only a small cohort of samples obtainable for evaluation. Well-differentiated HCC, which is reported to have lower levels of TA compared to poorly differentiated tumors [20] was not included in the samples. Furthermore, samples of cirrhotic liver and other chronic liver diseases as chronic hepatitis have not been assessed either. Evaluation of larger tissue series including all grades of HCC, and chronic liver diseases are needed before tRQ-TRAP could be ready for clinical application.

In summary, our analysis confirms other studies and demonstrates utility of tRQ-TRAP to quantify telomerase activity in clinical tissue samples without interference by PCR inhibitors.

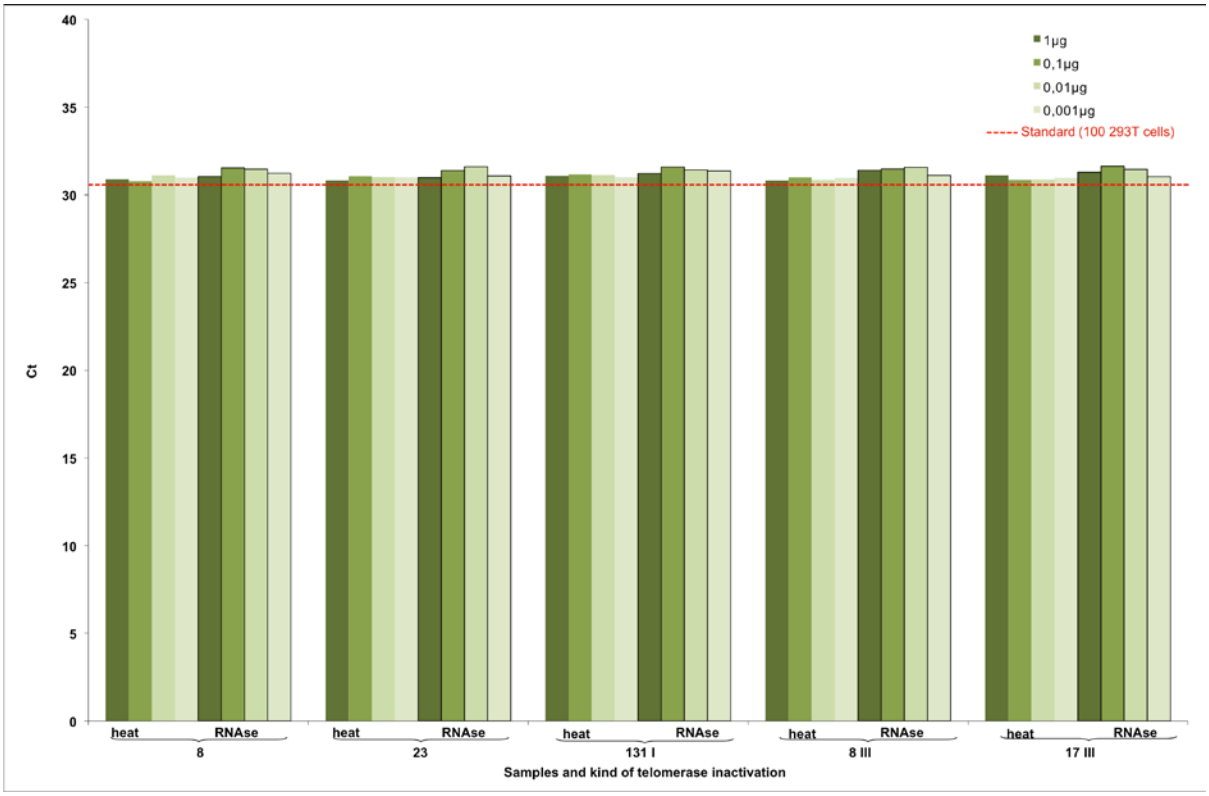

Figure 3: Samples were inactivated by heat-denaturation (70°C for 20 minutes) or digestion with ribonuclease A. Afterwards a standard containing 100 293T cells was spiked into every denaturated sample dilution and tRQ-TRAP assay was performed. There was no observable PCR inhibition independent of denaturation technique in all dilutions compared to the standard alone (depicted as dash line).

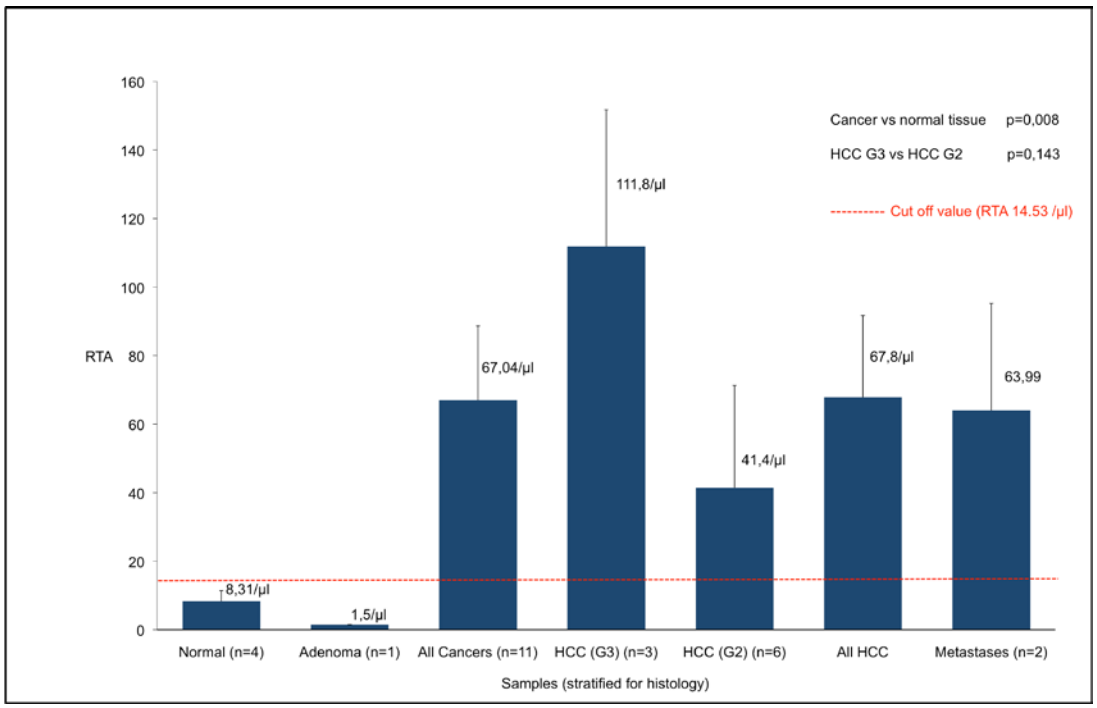

Figure 4: Relative telomerase activity per µg protein (RTA) was measured with the tRQ-TRAP and displayed as means ± SEM (error bars) for each sample. The data show elevated telomerase activity in hepatocellular carcinoma (G2 and G3 HCC) and liver metastases in comparison to normal liver samples and a benign liver lesion (sample 29 I, liver adenoma). The difference in telomerase activity between cancer samples (median RTA 78.92, *n* = 10) and normal liver samples (median RTA 8.03, *n* = 4) was significant (Mann-Whitney *U* test, *P* = 0.008). The depicted line displays the cut-off value for relevant RTA-levels (above mean RTA + 2 SEM of normal liver samples).

Conclusions

We optimized sample dilution and evaluated the significance of the amount of starting material for TRAP extraction as well as tissue inhibitors against Taq polymerase on the performance of our tRQ-TRAP. The optimized procedure includes assessment of RNA integrity and an inhibitor-control sample to identify false-negative results due to PCR inhibition. As shown by the assessment of human liver samples, tRQ-TRAP is an auspicious method to analyze clinical tissue samples without interference by high telomerase activity levels or the presence of Taq polymerase inhibitors. In contrast, evaluation of highly diluted samples in end-point assays, such as TeloTAGGG Telomerase PCR ELISA^{PLUS} and TRAPeze, would profoundly decrease sensitivity. Furthermore, results with the tRQ-TRAP can be obtained more rapidly and the closed-tube system diminishes the danger of carry-over contamination. Therefore, tRQ-TRAP is a sensitive and rapid method to quantify telomerase activity in clinical tissue samples. The assay may be employed to detect telomerase activity in the context of molecular diagnostics during carcinogenesis and to monitor telomerase activity during cancer progression and treatment.

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