Quantitative Analysis of Ornithine Decarboxylase mRNA by Reverse Transcription-PCR with the LightCycler System, Xavi Orta, Joaquin Balsells, Jorge Olsina, Yusmy Venereo, Toni Segovia-Silvestre, and Mireia Farriol*

Cycler System, mRNA by Reverse Transcription-PCR with the Light-Quantitative Analysis of Ornithine Decarboxylase

Polyamines originate from the decarboxylation of ornithine, which gives rise to putrescine, the first polyamine in this metabolic pathway. The reaction is catalyzed by ornithine decarboxylase (ODC; EC 4.1.1.17), a rate-limiting enzyme in polyamine synthesis. The importance of the regulation of this pathway in colon neoplasm has been elucidated, but the application of ODC expression to clinical diagnosis as a prognostic marker has not been completely defined (1–3). To be used for this purpose, the method for measuring ODC mRNA should be sensitive, accurate, and require only small samples of colon or tumor tissue. With recent innovations in PCR technology, these analytical requirements can now be achieved (4). A new thermal cycler (LightCycler; Roche) that combines continuous fluorescence monitoring with a rapid-cycle PCR within glass capillaries is now available (5), but has not yet been used in this context. This study describes a real-time reverse transcription (RT)-PCR assay based on LightCycler technology to quantify ODC mRNA. This method was successfully applied to the measurement of ODC mRNA in tumor and nontumor tissue samples from colon carcinoma patients.

We studied 18 tissue samples from nine patients with colon carcinoma (seven men and two women; mean ± SD age, 65.1 ± 7.8 years) who underwent surgical treatment. Samples of tumor tissue (T) and unaffected colon (nontumor; NT) weighing ~30 mg were obtained from the resected surgical specimen of each patient in the operating room and were immediately immersed in liquid nitrogen and stored at −80 °C until analysis. The study protocol was approved by the Vall d’Hebron Hospital Ethics Committee. The characteristics of the patients and histologic analyses of the tumor specimens are shown in Table 1. RNA isolation was performed with the Roche isolation reagent set (cat. no. 2033674) according to the manufacturer’s instructions. Samples were disrupted and homogenized in the presence of a strong denaturing buffer containing guanidine-HCl. After the addition of ethanol, the RNA in the sample was selectively bound to glass fiber fleece in the presence of a chaotropic salt. Residual contaminating DNA was digested by DNase I applied directly on the glass fiber fleece. After a series of rapid “wash-and-spin” steps to remove contaminating cellular components, RNA was eluted in nuclease-free water.

For real-time RT-PCR, the method combines rapid thermocycling with online fluorescence detection of PCR product formation as it occurs. Fluorescence monitoring of PCR amplification is based on the concept of fluorescence resonance energy transfer between two adjacent dyes. Fluorescein is used as the donor fluorophore and LC Red 640 as the acceptor fluorophore. Once conditions are established, the amount of fluorescence resulting from the two probes correlates with the amount of PCR product. For quantification purposes, the LightCycler software automatically identifies the first maximum of the second derivative curve of the function that relates fluorescence to cycle number, and this serves as the Cp in the second derivative maximum method. There is no user influence in this step, and the problem of between-run variations in fluorescence is obviated (no influence of background). The higher the starting copy number, the lower the Cp.

The amount of ODC mRNA in each sample was measured by use of the calibration curve prepared with an ODC RNA external standard synthesized in our laboratory. The porphobilinogen deaminase (PBGD) housekeep-

Table 1. Characteristics of patients and ODC expression results.

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age, years</th>
<th>Surgery</th>
<th>Tumor stage</th>
<th>Lymph node*</th>
<th>TNM stage</th>
<th>Differentiation grade</th>
<th>CEA, μg/L</th>
<th>CA 19.9, ng/mL</th>
<th>T</th>
<th>NT</th>
<th>T:NT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>67</td>
<td>RC</td>
<td>T1</td>
<td>N0 I</td>
<td>Well</td>
<td>2.9</td>
<td>11</td>
<td>608</td>
<td>830</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>64</td>
<td>RC</td>
<td>T3</td>
<td>N2 II</td>
<td>Poor</td>
<td>1.7</td>
<td>82</td>
<td>836</td>
<td>1142</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>52</td>
<td>LC</td>
<td>T3</td>
<td>N0 II</td>
<td>Moderate</td>
<td>7.4</td>
<td>199</td>
<td>525</td>
<td>42</td>
<td>12.50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>63</td>
<td>S</td>
<td>T3</td>
<td>N0 II</td>
<td>Moderate</td>
<td>1.6</td>
<td>10</td>
<td>208</td>
<td>137</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>68</td>
<td>S</td>
<td>T3</td>
<td>N1 IV</td>
<td>Moderate</td>
<td>5.6</td>
<td>432</td>
<td>1159</td>
<td>584</td>
<td>1.98</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>56</td>
<td>RC</td>
<td>T3</td>
<td>N2 IV</td>
<td>Moderate</td>
<td>5146</td>
<td>1463</td>
<td>348</td>
<td>57</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>69</td>
<td>RC</td>
<td>T2</td>
<td>N0 I</td>
<td>Well</td>
<td>2.7</td>
<td>264</td>
<td>367</td>
<td>99</td>
<td>3.71</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>79</td>
<td>RC</td>
<td>T3</td>
<td>N0 II</td>
<td>Well</td>
<td>0.8</td>
<td>161</td>
<td>321</td>
<td>170</td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>68</td>
<td>RC</td>
<td>T4</td>
<td>N0 II</td>
<td>Well</td>
<td>0.1</td>
<td>10</td>
<td>120</td>
<td>122</td>
<td>0.98</td>
<td></td>
</tr>
</tbody>
</table>

* N0, negative lymph node; N1, less than four positive lymph nodes; N2, more than four positive lymph nodes.

† CEA, carcinoembryonic antigen; RC, right colectomy; LC, left colectomy; S, sigmoidectomy.

‡ Reference values =< 6.0 μg/L.

§ Reference values <370 g/L.

¶ Copies of ODC normalized to copies of PBGD.

Mean ± SD.

Cl enical Chemistry 48, No. 10, 2002 1779
ing gene was used as endogenous control to compensate for different degrees of inhibition during reverse transcription and PCR (6). Mathematically, the result for each sample is expressed as the ratio of ODC to PBGD copy numbers, and this relative ODC expression in tumor tissue was divided by the relative ODC expression in nontumor tissue of the same patient. The final results were expressed as the tumor/nontumor ratio (T/N).

For the synthesis of an ODC RNA external standard, we used the forward primer 5'-TgAGCgTTACCTGATTTgAT-TgCTTTtgAAAACAT-3' and the reverse primer 5'-CgAAAATTAATACgACTCATAATAgggAgAAACACAT-TAATACTAgCCgAAgCAC-3'. The forward primer was designed to contain sequences for the Hpa restriction endonuclease site and the ODC gene, and the reverse primer was designed to contain sequences for the T7 promoter and the ODC gene. One-step RT-PCR was carried out on a LightCycler using 2.7x LC-RNA Master Hybridization Probes (5.5 μL) containing buffer, deoxynucleotide triphosphates, and Tth DNA polymerase; 3.25 mM Mn(OAc)₂; 0.5 μM each primer; 1 μL of RNA (0.5 μg of total RNA isolated from a colon tumor sample); and water to a final volume of 15 μL. Reverse transcription was performed at 61 °C for 20 min, followed by denaturation at 95 °C for 2 min and PCR amplification for 40 cycles (denaturation at 95 °C for 1 s, annealing at 65 °C for 15 s, and extension at 72 °C for 13 s). Product size was verified by 1.8% agarose gel electrophoresis. Amplified DNA was purified by silica adsorption to remove contaminating primers, nucleotides, salts, and proteins (High Pure PCR Product Purification Kit; cat. no. 1732668; Roche) and then digested with a final concentration of 1 U/25 μL HpaI (Roche) at 37 °C for 1 h to provide a template for the transcription with a uniform 3' end. After purification, the digested DNA was used for in vitro transcription at 37 °C for 4 h with 20 U/20 μL T7 RNA polymerase (Roche).

RNA transcripts were treated with 72 U/40 μL DNase I to remove the DNA template and extracted with phenol-chloroform, followed by precipitation and washing with ethanol. The entire process was performed twice. Finally, the RNA was resuspended in nuclease-free water and quantified with the spectrophotometer. The ODC RNA internal standard was free of protein (A₂₆₀/₂₈₀ = 1.78), and DNA contamination was negligible as indicated by the differences between the Cp (27.19 vs 8.43 cycles) of two reactions both containing 2.22 × 10¹⁰ starting copies, one in the absence of reverse transcriptase and the other a complete RT-PCR, respectively. Transcript was stored at −80 °C in tubes containing 4.44 × 10¹¹ copies/μL. The PBGD RNA calibrator was obtained commercially (cat. no. 3146073; Roche).

In each run serial dilutions of both calibrators were amplified, and the Cp values were plotted against the logarithmic copy numbers. Subsequently, linear regression analysis was carried out to generate the calibration curve for each gene, averaging the data generated in five independent analytical runs over 5 different days. The total number of data points in these curves was 25 for ODC and 17 for PBGD.

One-step RT-PCR was optimized to quantify ODC and PBGD mRNA concentrations in parallel tubes with the LightCycler. The same amount of total RNA (from 100 to 690 ng) was used for all LightCycler capillaries for the same patient. The reaction mixture and RT-PCR conditions were identical to those described above for synthesis of the ODC mRNA calibrator with the following exceptions: the ODC primers used were forward, 5'-TgATTgtgATgCTTTtgAAAACAT-3', and reverse, 5'-ACACAT-TAATACTAgCCgAAgCAC-3'. The ODC probes were fluorescein-5'-TgAAATTgCTgATgTgCCAC-3' and Red 640-5'-TgCCCTgACATCACATgTAATCgTCgC-3' (final concentration, 0.2 μM), and PCR amplification used an annealing temperature of 57 °C.

The total time required for RNA preparation and a LightCycler RT-PCR was ~1.75 h. Assay linearity covered at least five orders of magnitude for ODC (Fig. 1) and three orders of magnitude for PBGD, with concentration ranges (number of copies/μL) of 4.44 × 10⁴ to 4.44 × 10⁴ and 1 × 10⁴ to 1 × 10⁴, respectively. The Cps of samples were 16.0–24.0 cycles for ODC and 31.6–36.6 cycles for PBGD, indicating a dissimilar expression for these genes. PBGD mRNA belongs to a low-abundance class mRNA (250–750 copies/ng of RNA), and ODC mRNA in colon tissue belongs to a high-abundance class mRNA (>50 000 copies/ng of RNA).

Sample results were calculated from the equations: 
\[ y = -2.72x + 45.53 (r = -0.999) \text{ for PBGD} \]
\[ y = -3.68x + 45.64 (r = -0.999) \text{ for ODC, corresponding to the calibration curves of the two genes.} \]

For the ODC calibration curve, the confidence intervals for the slope and y-intercept, respectively, were −3.52 to −3.87 and 4.33–46.95 cycles. For the PBGD calibration curve, they were −2.24 to −3.51 and 44.10–50.59 cycles, respectively.

The imprecision of the method was established through the CV of the ratio of copy numbers of ODC to PBGD. The CV determined with the calibration curve using the duplicate assay values obtained in separate assays in five patients was 14%. When the ratio of the same duplicate assay values was calculated with the daily curve, the CV was substantially higher, at 30%.

![Fig. 1. Calibration curve used for ODC mRNA quantification.](https://academic.oup.com/clinchem/article-abstract/48/10/1779/5642316) Symbols represent individual experiments. \( r = -0.999 \).
The relative ODC mRNA concentration, expressed as number of copies, was higher in tumor tissue than in nontumor tissue (1.41-fold) and showed great variability in both tissues. We observed that in six of nine cases (67%), tumor to nontumor ratios (T:NT) for ODC mRNA expression were >1 (mean T:NT ratio, 3.3). Patients 3, 6, and 7 (33%) showed the most markedly increased ratios (Table 1). Examination of our results, expressed as number of copies of ODC mRNA, showed a notable overlapping of the range of values obtained in the two tissues. For this reason, overexpression cannot be determined by comparing a tumor tissue value with the mean value of all the nontumor samples. However, the ODC mRNA T:NT ratio may be used to establish associations with other prognostic markers (e.g., tumor histology, sex, and age). For example, the two patients with the highest ratios (patients 3 and 6) presented several negative prognostic markers, including age, location of the lesion, metastasis, and plasma tumor markers (Table 1).

Our results obtained with LightCycler technology, expressed as the T:NT ratio (3.35 ± 3.84), were similar to data obtained by Yoshida et al. (2.1 ± 0.9) (2) and Radford et al. (4.19 ± 2.76) (3), using Northern blot analysis. Moreover, the percentage of cases with a ratio >1 (67%) was also in keeping with the results reported by these authors, supporting that increased ODC mRNA may represent an important feature of the neoplastic state in the colon.

In conclusion, the real-time RT-PCR assay for ODC mRNA quantification using the PBGD housekeeping gene as an internal control is a fast and highly reproducible method that is suitable for analysis of small tissue samples. Its ease makes it applicable to large series of patient samples as a routine laboratory method. We believe that studies in larger series with the method described here could provide definitive results as to the role of ODC expression in colon neoplasia.

We thank Dr. Carmen Ricós for suggestions regarding quality control of the method, Roche Diagnostics for technical support, and Celine Cavallo for linguistic advice.

Stability of Novel Plasma Markers Associated with Cardiovascular Disease: Processing within 36 Hours of Specimen Collection, Jennifer K. Pai,1,3* Gary C. Curhan,1,5 Carolyn C. Cannuscio,2,4 Paul M. Ridker,3,4 and Eric B. Rimm1,2,5 (Departments of 1 Epidemiology and Nutrition, Harvard School of Public Health, Boston, MA 02115; Divisions of 2 Preventive Medicine and 4 Cardiology, 5 Channing Laboratory, Department of Medicine, and 6 Center for Cardiovascular Disease Prevention, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115; Merck & Co., Inc., West Point, PA 19422; Department of Pathology, Children’s Hospital Medical Center and Harvard Medical School, Boston, MA 02115; * address correspondence to this author at: Harvard School of Public Health, Department of Epidemiology, 677 Huntington Ave., 9th Floor, Kresge Bldg., Boston, MA 02115; fax 617-566-7805, e-mail jpai@hsph.harvard.edu)

Plasma markers are ideally measured prospectively because marker concentrations may change after diagnosis with disease. In many large prospective studies, such as the Nurses’ Health Study (1) and Health Professionals Follow-up Study (2), blood samples are collected, placed on ice, and mailed back to the central laboratory for processing via overnight or next-day mail service. Because economic constraints typically lead researchers to collect only one sample per study participant (3, 4), it is important to determine the time frame within which markers remain stable and to optimize the methods for handling and processing the sample. Although many studies have examined marker stability after long-term storage, few studies have assessed the impact of transport conditions on whole blood not immediately processed.

We selected a series of lipid and novel inflammatory markers whose concentrations have been shown or have been suspected to influence the risk of cardiovascular disease: C-reactive protein (CRP), fibrinogen, lipoprotein (a) [Lp(a)], apolipoprotein B (apoB), intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), p-selectin, von Willebrand factor (vWF), oxidized LDL (oxLDL), anti-cardiolipin antibodies (aCLAbs), tumor necrosis factor receptors I and II (TNF-RI and TNF-RII), and matrix metalloproteinase-1 (MMP-1) (5–13). The purpose of this study was to evaluate the stability of selected novel markers of cardiovascular disease under time and temperature conditions that simulated sample transport by mail for up to 36 h before processing.

We included 17 premenopausal women, 25–45 years of age, who had responded to a recruitment advertisement. Blood samples were collected from each woman into three 15-mL Vacutainers containing sodium heparin and placed on ice until processing. The time to processing was defined as the time from when the samples were first placed on ice until they were centrifuged to separate the plasma from the cells, aliquoted into 2-mL tubes, and finally stored in liquid nitrogen (−140 °C) until analysis. One sample was processed immediately and defined as the baseline at 0 h. The second and third samples were

References