Analytical Biochemistry 398 (2010) 266–268

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Notes & Tips

# Quantification of epidermal growth factor receptor T790M mutant transcripts in lung cancer cells by real-time reverse transcriptase–quantitative polymerase chain reaction

Gang Chen [a,b], Peter Kronenberger [a,c], Ijeoma Adaku Umelo [a], Erik Teugels [a], Jacques De Grève [a,*]

[a] Laboratory of Molecular Oncology and Department of Medical Oncology, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel, 1090 Brussels, Belgium
[b] Department of Pathology, Guangxi Medical University, Nanning, Guangxi 530021, People's Republic of China
[c] Laboratory for Biotechnology, Departement Gezondheidszorg, Erasmushogeschool Brussel, 1000 Brussels, Belgium

ARTICLE INFO

*Article history:*
Received 11 September 2009
Received in revised form 26 November 2009
Accepted 30 November 2009
Available online 2 December 2009

ABSTRACT

A simple and sensitive real-time reverse transcriptase–quantitative polymerase chain reaction (RT–qPCR) was developed to quantify threonine-to-methionine substitution at amino acid position 790 (T790M) mutant transcripts in a wild-type (wt) epidermal growth factor receptor background. The assay is based on three unmodified oligonucleotides, and both SYBR Green and a Taqman probe can be used. To increase the discrimination between mutant and wt signals, ARMS (amplification refractory mutation system) and LNA (locked nucleic acid) primers were tested, but a benefit was observed only with plasmids and not with cellular complementary DNA. The RT–qPCR assay using transcript-specific primers can detect as few as 1% T790M transcripts in a wt background and, therefore, will be useful in RNA interference studies specifically targeting mutant RNA.



Some lung cancer patients are initially very responsive to small molecule tyrosine kinase inhibitors such as gefitinib and erlotinib. The sensitivity of the cancer cells is caused by sensitizing mutations in the epidermal growth factor receptor (EGFR).[1] However, the efficacy of the inhibitors is limited in time due to the appearance of cells with an additional resistance conferring mutation, often a threonine-to-methionine substitution at amino acid position 790 (T790M) located in the receptor tyrosine kinase domain [1]. Many groups have been searching for sensitive methods to detect the small fraction of T790M mutant copies in a wild-type (wt) context. Most assays rely on polymerase chain reaction (PCR) [2–7] and the ability of these assays to detect genomic mutations in wt background ranges from 1 to 25% sensitivity. Some methods claim still higher sensitivity, but these either require specialized equipment [8], employ conceptually complex processes that may be difficult to troubleshoot [9], or require restriction enzyme digestion [10] that is not easily implemented in a reverse transcriptase (RT)–PCR workflow. Moreover, all of these assays were designed to detect mutations in genomic DNA and are based on specific primer sequences that are located within exon 20 surrounding the T790M mutation. For RT–PCR, however, primers should be located on exon–exon borders or located in different exons to exclude coamplification of genomic sequences. The genomic methods, therefore, are not directly applicable to transcript detection.

Here we describe a variant of an allele-specific PCR that we more appropriately call "transcript-specific" RT–PCR (because the term "allele" now often refers to DNA sequence variants). In our method, total RNA is first converted to complementary DNA (cDNA) using a reverse primer that is common to both mutant and wt transcripts and is located on the border of exons 20 and 21 of EGFR. Mutant- and wt-specific sequences are subsequently amplified using transcript-specific reverse primers located in exon 20 that contain either a mutant or wt base at their 3′ end and with a common forward primer located in exon 19.

We initially designed different exon-spanning primer sets with the point mutation T790M located in either the forward or reverse primer and with the reverse primers used directly in cDNA synthesis. With this setup, however, the results were unsatisfactory with respect to both PCR efficiency and discrimination of wt and mutant signals (data not shown). Much better results were obtained when the cDNA synthesis was first performed using a common reverse primer, primer 2706R located on the exon 20/21 border (Table 1 and Fig. 1A), followed by PCR using wt- or mutant-specific primers. The best result in terms of PCR efficiency and discrimination, furthermore, was obtained when the reverse primer contained the mutation site. The wt version of this primer, unfortunately,

* Corresponding author. Fax: +32 2 4776210.
*E-mail address:* jacques.degreve@uzbrussel.be (J. De Grève).

[1] *Abbreviations used:* EGFR, epidermal growth factor receptor; T790M, threonine-to-methionine substitution at amino acid position 790; wt, wild-type; PCR, polymerase chain reaction; RT, reverse transcriptase; cDNA, complementary DNA; ARMS, amplification refractory mutation system; LNA, locked nucleic acid; MCS, multiple cloning site; pCMV, cytomegalovirus promoter; $C_t$, threshold cycle; MMLV, Moloney murine leukemia virus; qPCR, quantitative PCR; CV, coefficient of variation.

0003-2697/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2009.11.034

**Table 1**
Primers and probe sequences for EGFR T790M mutant transcripts by real-time RT–qPCR.

| | | Forward primer | Sequence | Location | Reverse primer | Sequence | Location |
|---|---|---|---|---|---|---|---|
| Allele-specific primers | T790M locates at reverse primer | Primer 2510F[a] | CCAACAAGGAAATCCTCGAT | Exon 19, nt 2510–2529 | Primer 2615R[a] | AGGGCATGAGCTGC***A*** | Exon 20, nt 2615–2629 |
| | | Primer 2525F | TCGATGAAGCCTACGTGATG | Exon 19/20, nt 2525–2544 | T790M | | |
| | | Primer 2496F | AACATCTCCGAAAGCCAACAAG | Exon 19, nt 2496–2517 | | | |
| | | Primer 2412F | GTTCGGCACGGTGTATAAGG | Exon 18/19, nt 2412–2431 | | | |
| | T790M locates at forward primer | Primer 2599F T790M | ACCGTGCAGCTCATCAT | Exon 20, nt 2599–2615 | Primer 2706R | GATCGCAAAG GGCATGAA | Exon 20/21, nt 2706–2723 |
| | | Primer 2598F T790M | CACCGTGCAGCTCATCA***T*** | Exon 20, nt 2598–2615 | Primer 2696R | GGTGTGTGCAG ATCGCAAA | Exon 20, nt 2696–2714 |
| | | | | | Primer 2772R | CGTACTGGTGA AAACACCG | Exon 21, nt 2772–2790 |
| ARMS | | Primer 2510F[a] | CCAACAAGGAAATCCTCGA***T*** | Exon 19, nt 2510–2529 | Primer 2615R T790M ARMS 1 | AGGGCATGAGCTG***AA*** | Exon 20, nt 2615–2629 |
| | | | | | Primer 2615R T790M ARMS 2 | AGGGCATGAGCT***TC****A* | Exon 20, nt 2615–2629 |
| | | | | | Primer 2615R T790M ARMS 3 | AGGGCATGAGC***A***GC***A*** | Exon 20, nt 2615–2629 |
| LNA | | LNA 2510F | CCAACAAGGAAATCCTCGAT | Exon 19, nt 2510–2529 | LNA 2615R T790M | AGGGCATGAGCTGC***A*** | Exon 20, nt 2615–2629 |
| Probe | | Probe 2569 | FAM-TGCCGCCTGCTGGGCAT | Exon 20, nt 2569–2585 | | | |

[a] The allele-specific primer set gaining the best PCR efficiency and largest discrimination, which was performed with the ARMS and LNA techniques. Bold italicized letters identify mutations.

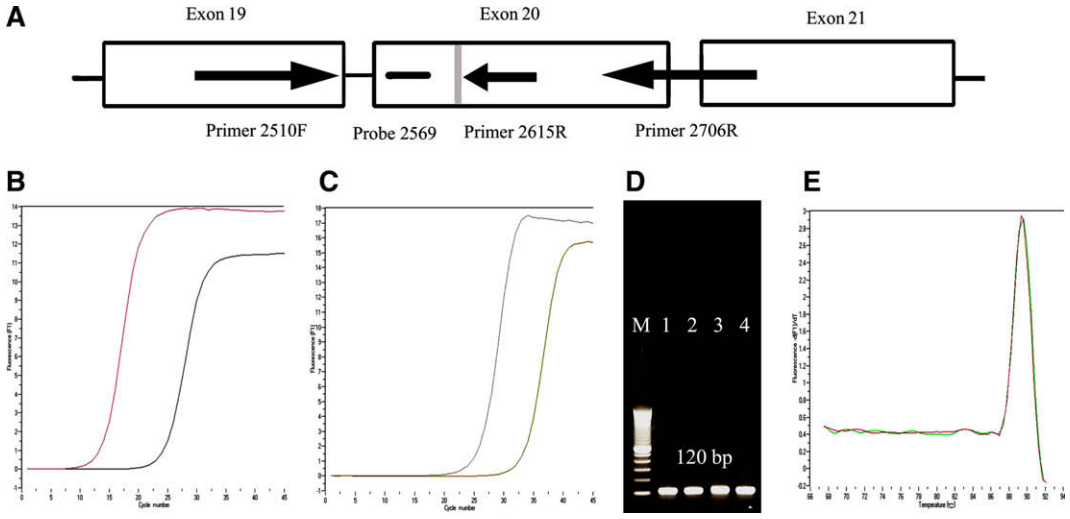


**Fig. 1.** Primers and probe locations, amplification curves, electrophoresis gel, and melting curves of allele-specific primers for EGFR T790M mutant transcripts detection by real-time RT–qPCR with SYBR Green. (A) The schematic shows the EGFR gene exon (boxes) and intron (lines) structure and locations of real-time RT–qPCR primers and probe: primer 2510F (CCAACAAGGAAATCCTCGAT, nt 2510–2529); primer 2615R T790M mutation (AGGGCATGAGCTGCA, nt 2615–2629); primer 2706R (TTCATGCCCTTTGCGATC, nt 2706–2723, sitting on the border of exon 20 and exon 21); probe 2569 (FAM-TGCCGCCTGCTGGGCAT, nt 2569–2585, NM_005228.3, 5599 bp). (B) Amplification curves with plasmids. The $C_t$ value with T790M mutation plasmid was 13.90 and with wt plasmid was 24.64 ($\Delta C_t$ = 10.74). (C) Amplification curves with cellular cDNA. The $C_t$ value with cDNA from H1975 was 25.96 and with cDNA from H358 was 33.49 ($\Delta C_t$ = 7.53). (D) Electrophoresis gel showed a single band for T790M transcript amplification. Lane M: DNA marker; lane 1: cDNA from H358; lane 2: cDNA from H1975; lane 3: wt plasmid; lane 4: T790M mutation plasmid. (E) Melting curves showed a single peak. Red curve: cDNA from H1975; Green curve: T790M mutation plasmid. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

produced a suboptimal PCR efficiency and specificity (data not shown). In this article, therefore, we focus on the mutant primer set 2510F and 2615R (T790M) (Table 1 and Fig. 1A).

We also tested this setup using ARMS (amplification refractory mutation system) primers that include additional mismatches and using primers that contain a 3′ LNA (locked nucleic acid) residue. Both modifications aim at increasing the difference in melting temperature of the matched and unmatched primers. For ARMS primers, extra mismatches were introduced at different nucleotides (first, second, and third nucleotides adjacent to the T790M mutation) 3′ to the primer (Table 1). It was found that the ARMS primer with the third nucleotide mismatch gained the best PCR efficiency and mismatch discrimination (data not shown).

We compared the three types of primers (transcript specific, ARMS, and LNA) on plasmid templates containing mutant and wt EGFR cDNA. The plasmids bearing an expression cassette for EGFR–wt (kindly provided by J. Jiang, Dana-Farber Cancer Institute) were derived from the backbone of the 5.47-kb pCIneo mammalian expression vector (Promega). The 3.64-kb cDNA sequence encoding EGFR–wt was cloned into the multiple cloning site (MCS) between *Xho*1 and *Nhe*1 of the pCIneo plasmid and driven by the cytomegalovirus promoter (pCMV). The EGFR point mutation T790M in exon 20 was introduced into the tyrosine kinase domain of the EGFR coding sequence in the pCIneo expression vector through the use of a GeneTailor Site-Directed Mutagenesis System (Invitrogen). Direct sequencing of the purified PCR products was performed using the ABI DNA sequencer (Applied Biosystems).

Real-time PCR was performed in the LightCycler 1.5 instrument, initially with SYBR Green detection. Plasmid (250 pg) was amplified with the LightCycler FastStart DNA Master$^{PLUS}$ SYBR Green

kit (Roche). Each 20-μl PCR reaction contained 4 μl of master mix, 2 μl each of forward and reverse primers 10× concentrated (500 nmol/L), 1 μl of template, and 11 μl of PCR-grade water. The magnesium chloride concentration was 3.0 nmol/L. After a 10-min hot start at 95 °C, PCR cycles were programmed with a denaturation at 95 °C for 10 s, annealing at 60 °C for 5 s, and extension at 72 °C for 5 s. Amplification was followed by melting curve analysis and gel electrophoresis to detect primer dimers and other nonspecific products (see Figs. 1D and 1E for transcript-specific primers [other figures not shown]).

The differences between the threshold cycle ($C_t$) values for wt and mutant plasmid ($\Delta C_t$) were 10.74 (using transcript-specific primers [Fig. 1B]), 12.87 (using ARMS primers), and more than 16.99 (using LNA primers). The large $\Delta C_t$ value of more than 16.99 obtained with LNA primers would suggest that mutant transcripts could be quantifiable in a $2^{16.99}$ = 130,166.62-fold excess of wt transcripts.

However, the results turned out to be quite different when analyzing cDNA from human non-small cell lung cancer cell lines NCI-H358 (for wt transcripts) and NCI-H1975 (these cells contain both mutant and wt transcripts). Cellular RNA was first isolated on the ABI PRISM 6100 PrepStation using the AbsoluteRNA solution containing DNase1 (Applied Biosystems) to remove contaminating DNA and PCR inhibitory substances. Cellular RNA (200 ng) was converted to cDNA with the common nondiscriminating but EGFR-specific primer (primer 2706R; for sequence, see Table 1) and using Moloney murine leukemia virus (MMLV) RT (Eurogentec) in a total volume of 10 μl. The reverse transcription step was performed on an Applied Biosystems ThermoCycler at 25 °C for 10 min for the initial step, 30 min at 48 °C for the reverse transcription step, and 95 °C for 5 min to inactivate the reverse transcription enzyme.

Although discrimination of mutant transcripts from wt transcripts was again possible using transcript-specific primers ($\Delta C_t$ = 7.53 [Fig. 1C]) and ARMS primers ($\Delta C_t$ = 9.12), the PCR efficiency was severely reduced using ARMS primers ($C_t$ = 28.47) and LNA primers ($C_t$ > 41) as compared with transcript-specific primers ($C_t$ = 25.96), and any discrimination power was consequently lost with LNA primers (data not shown). The behavior of our ARMS and LNA primers may be a consequence of the much higher complexity of the total cellular cDNA preparation when compared with the plasmids containing EGFR cDNA.

We were also interested in increasing the specificity of the RT–quantitative PCR (qPCR) by introducing a Taqman probe located in exon 20 (Fig. 1A; probe 2569; for sequence, see Table 1). The probe contained carboxyfluorescein at the 5′ end and a 3′ "black hole" quencher (BHQ-1, Eurogentec). Real-time PCR was performed using the LightCycler Taqman master mix (Roche). Using plasmid templates, the discrimination was slightly better with the Taqman probe ($\Delta C_t$ = 12.08) than with SYBR Green detection ($\Delta C_t$ = 10.74). Using cDNA from total cellular RNA, there was no significant difference (SYBR Green $\Delta C_t$ = 7.53 and Taqman $\Delta C_t$ = 7.33). The sensitivity of the Taqman assay was slightly lower, as evidenced by an increase of the $C_t$ value with approximately 1.56 cycles; the $C_t$ values on wt cellular cDNA were 27.52 and 25.96 in Taqman and SYBR Green assays, respectively, whereas on wt plasmids they were 15.88 and 13.90 in Taqman and SYBR Green assays, respectively).

To evaluate the ability of the assay to detect mutant cDNA in a wild-type background, we first prepared 10-fold serially diluted T790M plasmids in a constant amount of 250 fg of wt plasmid. Using SYBR Green detection, the mutant signal ($C_t$) increased linearly on dilution until the last dilution still above background, which was at 2.5 fg. This implies that an amount of 1% mutant plasmid can be detected. This sensitivity was confirmed using a higher background of 250 pg of wt plasmid and wt cDNA from H358 cells (data not shown). To further verify the specificity of the assay, we diluted the cDNA of H1975 cells with cDNA of normal peripheral blood nuclear cells to provide mixtures of 100%, 10%, 1%, 0.1%, 0.01%, and 0.001% mutant cDNA in wt background. The T790M mutant transcript could still be detected with a ratio of 1:100 (data not shown). To assess the detection limit of the assay, cDNA of H1975 cells was also diluted with distilled water to provide mixtures of 10, 1, 0.1, 0.01, 0.001, and 0.0001 ng of cDNA (mRNA equivalent). At least 100 pg of mutant cellular cDNA could still be detected (data not shown). The intraassay reproducibility proved to be high; the $C_t$ value was 27.12 ± 0.38 (mean ± SD) with a coefficient of variation (CV) of 1% in 10 independent measurements for the same cDNA sample. We also tested the interassay reproducibility with different RNA samples; for 30 independent measurements, the $C_t$ value was 27.05 ± 0.58 with a CV of 2% (data not shown). The quantification of the number of mutant transcripts in cDNA from cells can be based on the $C_t$ values of the pure mutant plasmids of which the molecular weight is known and, hence, the number of EGFR copies.

In conclusion, we have developed a simple transcript-specific RT–PCR with a sensitivity of 1% to specifically detect and quantify T790M mutant EGFR transcripts using either the SYBR Green detection format or a Taqman probe. Transcripts harboring the T790M resistance mutation can now be monitored in biopsies of lung cancer patients and in culture-grown lung cancer cells subjected to specific T790M targeted treatment by RNA interference.

## Acknowledgments

This study was supported by grants from the Belgium National Cancer Plan (NKP-29-011), the Stichting Tegen Kanker (Belgium), the Chinese Scholarship Council (CSC), and the Vrije Universiteit Brussel (VUB) PhD Programme. The authors thank Geert Meersseman for helpful suggestions on probe design and Goele Van Hassel for technical assistance.

## References

[1] W. Pao, V.A. Miller, K.A. Politi, G.J. Riely, R. Somwar, M.F. Zakowski, M.G. Kris, H. Varmus, Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain, PLoS Med. 2 (2005) e73.
[2] J.Y. Shih, C.H. Gow, P.C. Yang, EGFR mutation conferring primary resistance to gefitinib in non-small-cell lung cancer, N. Engl. J. Med. 353 (2005) 207–208.
[3] J. Li, L. Wang, P.A. Jänne, G.M. Makrigiorgos, Coamplification at lower denaturation temperature-PCR increases mutation-detection selectivity of Taqman-based real-time PCR, Clin. Chem. 55 (2009) 748–756.
[4] T. Kosaka, Y. Yatabe, H. Endoh, K. Yoshida, T. Hida, M. Tsuboi, H. Tada, H. Kuwano, T. Mitsudomi, Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib, Clin. Cancer Res. 12 (2006) 5764–5769.
[5] Y. Yatabe, T. Hida, Y. Horio, T. Kosaka, T. Takahashi, T. Mitsudomi, A rapid, sensitive assay to detect EGFR mutation in small biopsy specimens from lung cancer, J. Mol. Diagn. 8 (2006) 335–341.
[6] M. Inukai, S. Toyooka, S. Ito, H. Asano, S. Ichihara, J. Soh, H. Suehisa, M. Ouchida, K. Aoe, M. Aoe, K. Kiura, N. Shimizu, H. Date, Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer, Cancer Res. 66 (2006) 7854–7858.
[7] H. Miyazawa, T. Tanaka, Y. Nagai, M. Matsuoka, Huqun, A. Sutani, K. Udagawa, J. Zhang, T. Hirama, Y. Murayama, N. Koyama, K. Ikebuchi, M. Nagata, M. Kanazawa, T. Nukiwa, S. Takenoshita, K. Kobayashi, K. Hagiwara, Peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp-based detection test for gefitinib-refractory T790M epidermal growth factor receptor mutation, Cancer Sci. 99 (2008) 595–600.
[8] R.K. Thomas, H. Greulich, Y. Yuza, J.C. Lee, T. Tengs, W. Feng, T.H. Chen, E. Nickerson, J. Simons, M. Egholm, J.M. Rothberg, W.R. Sellers, M.L. Meyerson, Detection of oncogenic mutations in the EGFR gene in lung adenocarcinoma with differential sensitivity to EGFR tyrosine kinase inhibitors, Cold Spring Harb. Symp. Quant. Biol. 70 (2005) 73–81.
[9] K. Hoshi, H. Takakura, Y. Mitani, K. Tatsumi, N. Momiyama, Y. Ichikawa, S. Togo, T. Miyagi, Y. Kawai, Y. Kogo, T. Kikuchi, C. Kato, T. Arakawa, S. Uno, P.E. Cizdziel, A. Lezhava, N. Ogawa, Y. Hayashizaki, H. Shimada, Rapid detection of epidermal growth factor receptor mutations in lung cancer by the SMart-Amplification Process, Clin. Cancer Res. 13 (2007) 4974–4983.
[10] H. Asano, S. Toyooka, M. Tokumo, K. Ichimura, K. Aoe, S. Ito, K. Tsukuda, M. Ouchida, M. Aoe, H. Katayama, A. Hiraki, K. Sugi, K. Kiura, H. Date, N. Shimizu, Detection of EGFR gene mutation in lung cancer by mutant-enriched polymerase chain reaction assay, Clin. Cancer Res. 12 (2006) 43–48.