Development of an alternative method for an absolute quantification of folate transporters in human tumors using bacterial artificial chromosome vector

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INTRODUCTION

Quantification of gene expression is particularly relevant in clinical contexts such as in cancer. For example, folate receptor (FR) is reported to be overexpressed in many tumors [1]. The physical significance of FR over-expression and its role in the folate transport in tumors are not clear, especially compared to human reduced folate carrier (hRFC) another known folate transporter [2]. The need for determining whether both transport systems are operational in all tumors requires an accurate method for simultaneously quantifying transcript levels.

We developed an alternative method using a bacterial artificial clone (BAC) vector. This method is based on a simple standard-curve Q-PCR using defined amounts of *E. coli* EC100 cells harboring a single copy of pBeloBAC11 for simultaneous quantification of the *hrfc* and *fr* transcripts. We have validated the method by first studying the reproducibility of the standard curves in comparison with the plasmid method, and subsequently applying the method to determine *hrfc* and *fr* transcript levels in tumor and matched adjacent normal tissue.

MATERIALS AND METHODS

E. coli strains were incubated at 37°C under agitation. Exponential phase cultures (~10^7 colony forming unit per ml) were prepared by diluting 1:200 overnight cultures in LB fresh medium and incubating at 37°C for 8 to 9 hrs. The pBeloBAC11 and pUC19 were extracted with Qiagen® plasmid purification midi kit (Qiagen). To construct the standard for the quantitation of both transporters, total RNA was isolated from HepG2 cells by using the Qiagen® RNeasy total RNA isolation kit. Reverse transcription was performed with the Omniscript® RT kit (Qiagen), and cDNA obtained was used as a template for PCR amplifications.

As a first step, two PCR fragments were independently generated (Fig. 1) : (i) *hrfc1* 301 bp-length fragment with primer R1 (5'-TAAAGGATCCGGCGCCATCACGTC-3') containing a *Bam*HI recognition site at the 5' end (underlined bases), and R2 (5'-AATAGAATTCTGAAAGTGATGATGGTCTTG-3') containing an *Eco*RI recognition site at the 5'end (underlined bases); and (ii) *fr* 301 bp-length fragment with primer F1 (5'-ATAAGAATTCCAAAGAGGACTGTGAGCAAT-3') containing an *Eco*RI recognition site at the 5' end (underlined bases), and F2 (5'-AAGAAAGCTTCCCACTCATGGCTGC-3') containing a *Hin*dIII recognition site at the 5'end (underlined bases). Amplicons were digested with *Eco*RI then ligated to form a 608 bp fragment.

The second step consisted of a PCR with R1 and F2 in order to amplify the 608 bp fragment. The resulting fragment was digested with *Bam*HI and *Hin*dII, and cloned into pBeloBac11 vector. The recombinant BAC was designated as pAAN1 and transformed into *E. coli* EC100.

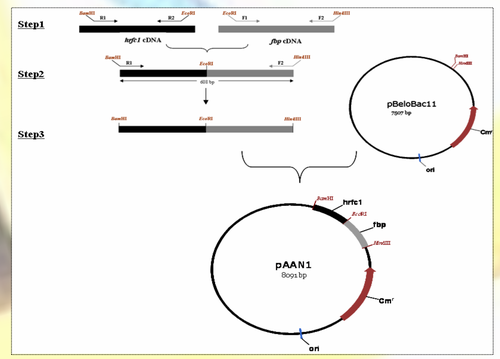

Fig. 1. Steps for the construction of the pAAN1 BAC vector, which contains 301 bp transcripts of each folate transporters (*hrfc1* and *fr*). Ori, origin of replication; Cm', chloramphenicol resistance gene. Note that sequences encoding *hrfc1*, *fr* genes and chloramphenicol resistance gene are not drawn to scale.

For plasmid vector construction, the same 608 bp-length fragment was cloned in a pUC19 vector using the method described for the BAC. The concentration of the plasmid pHH1 was measured using an Agilent 2100 Bioanalyzer.

The corresponding copy number was calculated using a previously developed equation [3]. High-quality, full length cDNA ready for gene amplification was purchased from BioChain Institute (Hayward, USA). cDNA samples were human primary tumor and normal matched pair tissue from the same individual. Tissues were human kidney (Cat#. C8235142-PP), lung (Cat#. C8235152-PP) and liver (Cat# C8235149-PP). The *hrfc1* and *fr* transcript levels in cDNA samples were quantified by PCR using the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Les Ulisses, France). Primers and fluorogenic probes (Table 1) were designed with *Primer Express Software version 2* (Applied Biosystems), and obtained from the same company.

The ANOVA Fisher's test was performed for statistical evaluation of mean values ± standard deviation for *hrfc1* and *fr* levels, with significance set at $P<0.05$. The *hrfc*/*fr* ratio was analyzed by using the ANOVA Games Howell post hoc test.

Table 1. Details of primers and probes used in Q-PCR to amplify *hrfc1* and *fbp*

Target gene	Genbank accession #	Primers and probe	Primers and probe sequences (5'-3')	% GC	Tm (°C)	Amplicon size (bp)
hrfc1	NM_003056	RFC-F	CCGCGGCTCCTACCAGTT	67	60	99
		RFC-R	AACGTGTTGACCCCGAAGAC	55	58	
		RFC MGB probe	6FAM-CGTGCCCATCGCCACCT	71	74	
fbp	NM_016731	FBP-F	TGGCACAAGGGCTGGAA	59	58	114
		FBP-R	CCAGATTTCATTGCACAGAACAG	43	58	
		FBP MGB probe	6FAM-TGGACTTCAGGGTTTAACA	42	71	

RESULTS AND DISCUSSION

Figure 2 shows the standard curves for *hrfc1* and *fr*. The dynamic range was wide, ranging from 10^2 to 10^7 copies. The pAAN1 linearity R^2 coefficient of C_t was found to be 0.99. For the pAAN1 vector containing 301 bp- sequences of both *hrfc* and *fr* cDNA, efficiency of the reactions (E) was calculated by $E = 10^{(1/m)}-1$, where *m* is the slope of the standard curve, indicating that Q-PCR efficiencies were 0.991 (CV=0.09%) and 0.992 (0.06%) for *hrfc1* and *fr*, respectively.

To test the reproducibility of alternative method, three different vector extractions were amplified separately using both *hrfc*-set and *fr*-set. Standard curves obtained for each assay were highly reproducible with no significant differences in slopes and intercepts between these three independent assays. We compared our method to the classical method, which uses both a plasmid vector (generally a high copy-number plasmid) and DNA quantification by spectrophotometer.

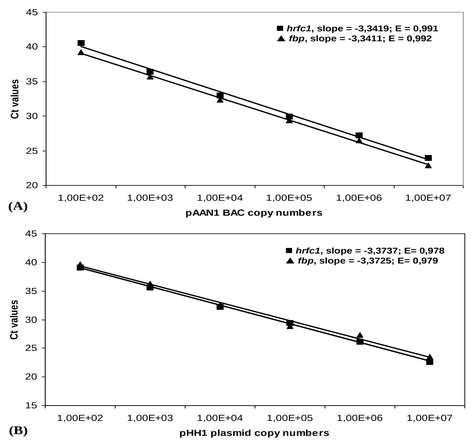

Fig. 2. Comparison of pAAN1 BAC and pHH1 plasmid standard assays. The standard curves were constructed with serial 10-fold dilutions of the pAAN1 (A) and pHH1 (B), ranging from 1×10^2 to 1×10^7 copies. Each standard dilution was amplified by Q-PCR using *hrfc1*- and *fr*-set in triplicate. For each gene, determined C_t v. alues were plotted against the logarithm of their known initial copy numbers. The standard curve was generated by linear regression through these points. The slopes and intercepts did not differ significantly (ANOVA test). (B) Plots of three standard curves generated from amplification with *hrfc1*-set in 3 separate assays. (C) Plots of three standard curves generated from amplification with *fr*-set in 3 separate assays.

Curves obtained from both methods were linear in the ranges tested by triplicate reactions. Standard curves obtained from specific *hrfc* and *fr* amplification of pAAN1 and pHH1 vectors did not differ significantly (Fig. 3).

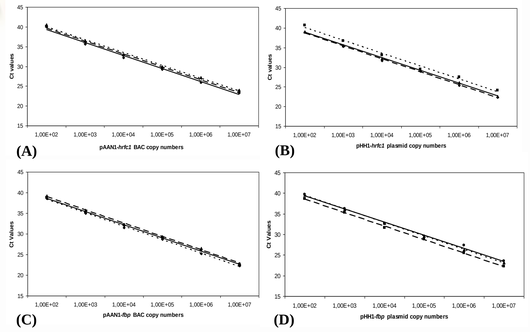

Fig. 3. pAAN1 BAC and pHH1 plasmid standard assay reproducibility. (A) pAAN1 BAC and (B) pHH1 plasmid plots of three standard curves generated from amplification with *hrfc1*-set in 3 separate assays. (C) pAAN1 BAC and (D) pHH1 plasmid Plots of three standard curves generated from amplification with *fr*-set in 3 separate assays. The slopes and intercepts did not differ significantly.

Results of this comparison confirm the pAAN1 copy number obtained as described above. The use of BAC method to quantify *hrfc* and *fr* indicated that both transporters have tissue specific expression patterns (Fig. 4). Finally, the *hrfc1*/*fr* ratio was found to be significantly increased in kidney and lung tumor tissues ($P>0.05$): (*i*) kidney 0.29 ± 0.06 in tumor *vs.* 0.05 ± 0.01 in normal tissue, (*ii*) lung 222.50± 43.09 in tumor *vs.* 0.69 ± 0.16 in normal tissue. The ratio did not differ significantly in liver tumor 3.45 ± 2.53 *vs.* 0.431 ± 0.159 in normal liver.

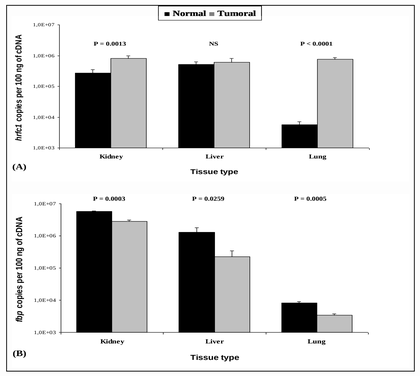

Fig. 4. Quantification of *hrfc1* (A) and *fr* (B) transcripts copies in different tumor tissues and in their adjacent normal tissues, using pAAN1. Data shown are means ± standard deviation, n = 4 per each tissue sample. *P* value indicates statistical confidence; NS (not significant).

In conclusion, the BAC method is an accurate method for absolute quantification of a target gene. It is highly reproducible and may be used routinely without establishing new standard curves for each run. Future applications of this method could include simultaneous quantification of multiple variant transcripts of target genes.

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