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ScienceDirect
Analytical Biochemistry 368 (2007) 111–121
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

A universal plate format for increased throughput of assays that monitor multiple aminoacyl transfer RNA synthetase activities

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Received 9 March 2007
Available online 18 May 2007

Abstract

Aminoacyl transfer RNA (tRNA) synthetases are intensely studied enzymes because of their importance in the establishment of the genetic code and their connection to disease and medicine. During the advancement of this field, several assays were developed. Despite many innovations, the sensitivity, simplicity, and reliability of the radiometric assays (which were among the first to be developed) have ensured their continued use. Four activities are measured by these assays: active site titration, amino acid activation, aminoacylation, and posttransfer editing (deacylation). In an effort to maintain the advantage of these assays while enhancing throughput, reducing waste, and improving data quality, a universal 96-well filter plate format was developed. This format facilitates the assays for all four of the widely studied activities.

Keywords: Transfer RNA; Filter plate; Editing; Scintillation counting; Aminoacyl tRNA synthetase; ATP–PPi exchange; Aminoacylation; Charging

The discovery of the genetic code ranks as one of the greatest achievements of the last century. In 1957, Crick proposed that RNA adaptors could link information carried in genes to their corresponding gene products—proteins [1]. It was later discovered that (in most organisms) each of the canonical 20 amino acids (AAs)[1] could be covalently linked to a cognate transfer RNA (tRNA) that bears the anticodon triplet of the code corresponding to the particular AA [2–4]. The covalent linkage of AAs to the adaptor molecules (tRNAs) is catalyzed by a set of 20 universal enzymes—aminoacyl tRNA synthetases (one for each AA).

The awareness that aminoacyl tRNA synthetases (AARSs) establish the genetic code gave investigators ample motivation to pursue the molecular mechanism of these enzymes. During more recent years, attention has also been placed on their medical relevance through development of synthetase inhibitors as antimicrobial agents [5,6] and, even more recently, identification of expanded functions in mammals that link signal transduction pathways and control of gene expression to translation [7]. And even mild disruption of one of the canonical activities—editing for suppression of mistranslation—leads to neurodegeneration [8].

AARSs establish the genetic code via a two-step reaction:

$$\mathrm{AA} + \mathrm{ATP} + \mathrm{AARS} \rightarrow \mathrm{AARS(AA-AMP)} + \mathrm{PPi} \quad (1)$$

$$\mathrm{AARS(AA-AMP)} + \mathrm{tRNA} \rightarrow \mathrm{AA-tRNA} + \mathrm{AMP} + \mathrm{AARS}. \quad (2)$$

In the first step, a highly reactive aminoacyl adenylate (AA–AMP) is formed through condensing ATP with the carboxylate of the AA. The second reaction uses this activated species to esterify the AA to the 3′ end of the tRNA (AA–tRNA). The sum of Eqs. (1) and (2) results in the spe-

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[1] *Abbreviations used:* AA, amino acid; tRNA, transfer RNA; AARS, aminoacyl tRNA synthetase; AA–AMP, aminoacyl adenylate; AlaRS, alanyl–tRNA synthetase; LB, Luria–Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; cDNA, complementary DNA; β-ME, β-mercaptoethanol; PVDF, polyvinylidene fluoride; TCA, trichloroacetic acid.

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.05.013

cific attachment of AAs to their cognate tRNAs, thereby linking each AA to its triplet anticodon embedded within the tRNA.

Some AARSs are inherently error prone in the AA activation step [9,10]. Hydrolytic activities that clear misactivated AAs improve fidelity sharply [11–13] and thereby prevent mistranslation [8,14–16]. For instance, alanyl–tRNA synthetase (AlaRS) misactivates serine or glycine [17,18]. A domain distinct from the active site for catalysis of Eqs. (1) and (2) prevents mischarged tRNAs from being released for participation in translation [17–20]:

$$\mathrm{Ser-tRNA^{Ala}} + \mathrm{AlaRS} \rightarrow \mathrm{Ser} + \mathrm{tRNA^{Ala}} + \mathrm{AlaRS}. \quad (3)$$

Using a variety of procedures, each of the reactions of Eqs. (1)–(3) can be studied. Active site titration [21] and AA-dependent ATP–PPi exchange assays [22] monitor the reaction depicted by Eq. (1), whereas the reactions in Eqs. (2) and (3) are monitored by assays for aminoacylation [23] and deacylation [24], respectively. These assays serve to kinetically dissect the enzymatic activities of any tRNA synthetase.

Radiometric assays for AARSs were established in early work. The basis of most assays is a radiolabeled product that is captured on a solid support (e.g., filter paper, activated charcoal). Although these assays are sensitive and straightforward, they suffer from low throughput due to laborious washing steps and the lack of a multichannel format. In addition, for measurement of AA–tRNA, stubborn product adsorption on filter paper results in a time- and concentration-dependent release of product into the scintillant [25]. Furthermore, many AAs generate high background due to "sticking" of the free AA to the filter paper.

As a result of these drawbacks, many assays have been developed to replace traditional assays for AA activation (by AA-dependent ATP–PPi exchange), active site titration, aminoacylation, and editing (by deacylation of mischarged tRNA). Low throughput is a prime motivation to replace these assays, and also nonradiometric approaches are desired as replacements for traditional radioactivity-based methods. Innovations to replace the proven historical assays have included coupled spectrophotometric assays [26,27] and fluorescent assays [28]. Although many of the alternative approaches are innovative, they often lack the sensitivity (spectrophotometric) and simplicity of the historical radiometric assays.

Two existing formats offer sensitivity and also are amenable to a high-throughput format. One was developed by Wolfson and coworkers and overcomes the limitations of AA concentration that exists with conventional aminoacylation assays (the tRNA is labeled instead of the AA) [29]. Despite its great utility, particularly for rapid kinetic experiments [30], this format remains to be widely adopted, possibly because of the product processing steps. (It requires thin-layer chromatographic separation of reactants and quantitation by phosphorimaging.) Another method, the scintillation proximity approach [31], is perhaps the most useful, but for many laboratories the required beads are too costly.

With these considerations in mind, we were motivated to "renovate" the established historical assays while maintaining their basic principles. Specifically, we sought to enhance throughput while maintaining simplicity. All of these assays were developed with a wide range of users in mind. Each was validated on standard scintillation counters or a 96-well beta counter. Because these advances, although simple in appearance, facilitated greatly the progress of several ongoing projects in our laboratory, the details are given here in a practical format that should be amenable to widespread application.

Materials and methods

Materials

Table 1 gives a list of key materials together with vendor and catalog numbers and brief comments. Counting of single-sample vials was accomplished in a $\mathrm{Beckman_{LSC}}$ LSV 6500 scintillation counter or a PE 1450 MicroBeta Trilux counter for plates.

RNA preparation

tRNA (*Escherichia coli* $\mathrm{tRNA^{Ala(GGC)}}$ or *E. coli* $\mathrm{tRNA^{Thr}}$) was produced by in vivo transcription with plasmid pWW–$\mathrm{ectRNA^{Ala}}$ for the overproduction of *E. coli* $\mathrm{tRNA^{Ala(GGC)}}$ and pWFW1015 for *E. coli* $\mathrm{tRNA^{Thr}}$ (W. Waas and P. Schimmel, unpublished). A 100-ml overnight culture of pWW–$\mathrm{ectRNA^{Ala}}$ or pWFW1015 in DH5α in Luria–Bertani (LB)–ampicillin was diluted into 10 L of LB–ampicillin and grown (37 °C) to midlog phase. Induction of overexpressed tRNA was accomplished by the addition of isopropyl β-D-thiogalactopyranoside (IPTG, 667 μM) for 24 h. Cells were harvested and lysed by phenol (pH 4.7). Total RNA was precipitated with ethanol and resuspended in 90 ml buffer W5 (Clontech, Mountain View, CA, USA). The tRNA was isolated through use of a Nucleobond AX 10,000 GIGA column (Clontech), followed by ethanol precipitation. The quantity of $\mathrm{tRNA^{Ala}}$ at this stage represented more than 80% of the total tRNA (>1400 pmol alanine acceptance/A_{260}). Overproduction and purification of $\mathrm{tRNA^{Thr}}$ from cells harboring plasmid pWW–$\mathrm{EctRNA^{Thr}}$ was achieved similarly. Total bovine tRNA was purchased from EMD Biosciences (San Diego, CA, USA). To produce mischarged $\mathrm{tRNA^{Ala}}$, the overexpressed purified $\mathrm{tRNA^{Ala}}$ (13 μM) was aminoacylated in the presence of editing-deficient C666A/Q584H AlaRS (2 μM), [^{3}H]Ser (64 μM), ATP (100 μM), and the assay buffer (50 mM Hepes [pH 7.5], 20 mM KCl, 2 mM dithiothreitol [DTT], and 10 mM $MgCl_2$). Similarly, reactions were incubated for 30 min at room temperature, quenched with 0.3 M sodium acetate (pH 5.0), passed through a G25 microspin column (GE Healthcare, Piscataway, NJ, USA), ethanol precipitated, and resuspended in 10 mM sodium