

A Rapid Method for Analysis of cDNA Synthesis Using Ion-Pair Reversed-Phase High Performance Liquid Chromatography

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Abstract

We have developed a rapid, quantitative method for analysing the outcome of the first strand synthesis step in cDNA library preparation, yield and molecular weight range of the final cDNA products are determined after size fractionation. This method involves conventional cDNA library construction including all enzymatic steps usually required, but replaces radioactive labelling of nucleic acids with fluorescence detection. The separation and quantification steps all involve ion-pair reversed-phase high performance liquid chromatography (IP RP HPLC). This quantitative method replaces the use of autoradiography and size exclusion chromatography with combined ion-pair reversed-phase high performance liquid chromatography and in line fluorescence detection. The result of this approach is combination of speed with the generation of reproducible, high quality cDNA libraries.

Keywords: cDNA, library construction, IP RP HPLC, size fractionation

Introduction

The construction of high quality cDNA libraries is of fundamental importance in contemporary molecular biology, since such libraries play a critical role in the analysis of all aspects of gene expression. Several methods for the construction of cDNA libraries have been published (for an overview see Kimmel and Berger, 1987; Ying, 2004; Harbers, 2008) most of which involve a series of enzymatic reactions including first strand synthesis, which is primed by oligo-dT and catalysed by reverse transcriptase; second strand synthesis, catalysed by DNA polymerase I; end filling, catalysed by *Pfu* DNA polymerase, and finally DNA ligation (DNA ligase) into a vector which has often been dephosphorylated (Okayama and Berg, 1982; Gubler and Hoffman, 1983; Bashirdes and Lovett, 2001; Harbers, 2008).

A major problem with this multi-step procedure is the frequent failure, or sub optimal yield of one or more of the steps. In order to insure that all steps have been successful, careful monitoring at all stages is carried out by radiolabeling and autoradiography procedures, which normally take one or two days depending on the specific activity of the labelled

material. The other problem is that when a pool of fragments, differing in molecular weight by an order of magnitude, are used for construction of cDNA libraries, the smaller fragments are selectively cloned (Matin and Hornby, 2000; Sambrook and Russell, 2001) and therefore, there is a need for size based fractionation of the cDNA, which is routinely achieved by gel filtration. This prolonged exposure of the cDNA to radioactive nucleotides increases the probability of radiation damage and requires discontinuous analysis, which is usually carried out by gel electrophoresis and an ethidium bromide fluorescence assay, in which part of each fraction is lost.

Ion-pair reversed-phase high performance liquid chromatography (IP RP HPLC) is an applicable conventional method which is currently being used for the analysis of compounds with more than one ionizable group. Since it is hard to retain such compounds on non-polar stationary phases, an ion-pairing reagent has been added to the mobile phase in IP RP HPLC (Peng and Dansereau, 2001). IP RP HPLC has been widely used in pharmaceutical and molecular biology studies as a highly accurate assay system, for instance to quantitatively analyse the gene expression (Doris et al., 1998), to investigate

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the metabolism of antisense drugs (Wei et al., 2006), to separate and identify metabolites in cellular extracts (Kiefer et al., 2010), to determine several chemical components in drug agents (Kyriakidesa and Panderi, 2007; Breuzovska et al., 2010; Zhang, 2010) and for proteome analysis (Delmotte et al., 2007) and simultaneous determination of nucleotide sugars (Nakajima et al., 2010).

Here we have combined the qualitative and quantitative analysis of the first strand products and final cDNA products (before or after fractionation) with IP RP HPLC. As a result of this method, the preparation and quality control of cDNA synthesis, for example, for library construction is made more reproducible and control over cDNA fragment sizes is much improved.

Materials and Methods

The WAVE® Nucleic Acid Fragment Analysis System, the DNASep® cartridge (7.8 mm internal diameter and 50 mm length) and triethylammonium acetate (TEAA) were provided by Transgenomic, San Jose, USA. The ZAP Express cDNA synthesis kit and ZAP Express cDNA Gigapack III Gold Cloning kit were purchased from Stratagene. Oligo-dT primer was synthesized at University of Sheffield and labelled with 5'-Tetrachloro-Fluorescein Phosphoramidite (TET, Glen Research): 5'-GAGACTCGAGTTTTTTTTTTTTTTTTTTT-3'.

The chromatography was performed using a two eluent buffer system: buffer A consists of 0.1 M TEAA, pH 7.0 and buffer B consists of 0.1 M TEAA, pH 7.0 and 25% (V/V) acetonitrile (ACN) and the chromatograms were recorded using a fluorescence detector. The analysis was performed using the following gradient conditions at 50°C: flow rate 0.75 ml/min, 44 to 55% B in 3 min, to 65% B in 8 min, to 70% B in 5 min, to 100% B in 0.5 min, hold at 100% B for 0.5 min, to 44% B in 1 min and hold at 44% B for 1 min. For fractionation of double strand cDNA using the UV detector the following gradient was used at 50°C and with a flow rate of 0.75 ml/min: 35 to 55% B in 2 min, to 65% B in 8 min, hold at 65% B for 2 min, to 75% B in 5 min, hold at 75% B for 1 min, to 100% B in 1 min, hold at 100% B for 1 min and to 35% B in 1 min.

Chinese Hamster Ovary (CHO) cells were grown in T25 tissue culture plastic flasks (Corning Life Sciences) in a humidified, 95% air: 5% CO₂ atmosphere at 37°C. The medium was a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium, supplemented with 5% Foetal Bovine Serum and 2 mM glutamine (all from Sigma, Poole).

The medium contained 50 U/ml penicillin and 50 mg/ml streptomycin (Sigma, Poole). Total RNA was extracted from the cells using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. The messenger RNA (mRNA) was then purified from total RNA using MessageMaker kit (Gibco-BRL).

Results and Discussion

mRNA was primed in the first-strand synthesis reaction with a TET-labelled oligo-dT primer and was reverse transcribed using MMLV-RT using a ZAP express cDNA synthesis kit (Stratagene). In order to analyse first strand synthesis reaction, a small volume of the product was injected on to a DNASep® column and compared with the chromatogram of oligo-dT primer alone. As shown in Figure 1, a spectrum of cDNAs arises and, as expected the bulk of the first strand cDNAs are relatively short. The second strand synthesis was accomplished using DNA polymerase I, and the appropriate components following the Stratagene protocol, but 4 µl of Fluorogreen (Amersham) was added instead of [α -³²P]dGTP. *Eco*RI adapters were added to both ends of the double stranded cDNA, which was then subjected to *Xho*I digestion.

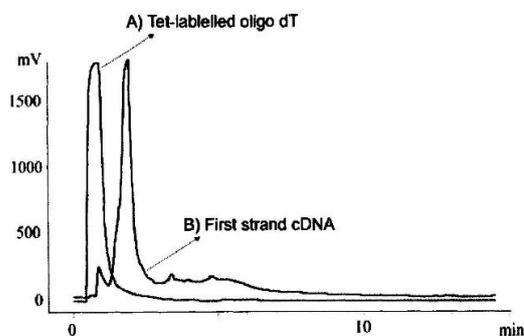


Figure 1. Chromatographic analysis of first strand cDNA synthesis.

Chromatogram A shows the result of the injection of TET-labelled oligo-dT and B shows the separation of 5 µl of the products of a first strand cDNA synthesis on a DNASep® column. The analysis was performed using a fluorescence detector with excitation and emission wavelengths set at 521 and 536 nm, respectively. The majority of products are short size single stranded DNAs that elute close to oligo-dT together with some longer products.

The cDNA was fractionated manually by gel filtration using a Sepharose CL-2B column according to the Stratagene's protocol, and a small

portion of each fraction was injected on to a DNASep® column to determine the molecular weight and yield of the fragments in each fraction as shown in Figure 2. This provides an example of the rapid analysis of cDNA synthesis products using IP RP HPLC. Other methods for analysis of cDNA size distribution including gel electrophoresis and autoradiography or digoxigenin labelling of second-strand synthesis reaction followed by blotting (Sambrook and Russell, 2001, Roeder, 1998) take considerably longer.

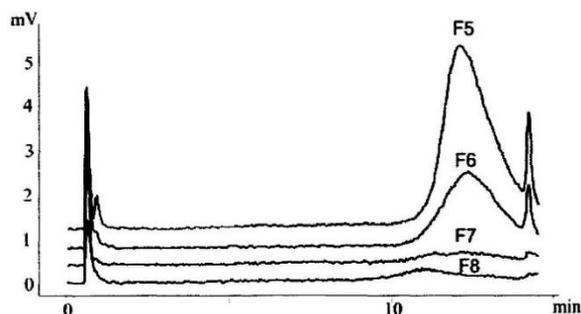


Figure 2. Rapid analysis of cDNA fractionation using IP RP HPLC. The double stranded cDNA was fractionated using a drip column (a standard gel filtration technique), according to Stratagene's manual and was subsequently analysed on the WAVE® system. 10 µl from 100 µl of each collected fraction was injected on to the DNASep® column and the chromatograms are related to fractions 5 to 8 (F5-F8) as indicated. Since Fluorogreen was incorporated into the second strand synthesis reaction the products were analysed using a fluorescence detector with excitation and emission wavelengths set at 494 and 525 nm, respectively. cDNAs greater than 500 bps elute after 12 minutes using a DNASep® column: most of the cDNAs are eluted in fractions 5 and 6.

In a separate experiment, the synthetic, double stranded cDNA was injected on to the DNASep® column (Figure 3), fractionated and collected. Those fractions containing larger fragments were selected and were used for the construction of a cDNA library which contained a larger than normal range of insert sizes as was also demonstrated before (Matin and Hornby, 2000; data not shown).

The generation of complete and full-length cDNA libraries is essential for functional assays of specific gene sequences. In this regard, the application of non-radioactive IP RP HPLC facilitates rapid quality control of cDNA synthesis reactions. This approach combines the advantages of conventional cDNA library synthesis protocols while eliminating most of their drawbacks.

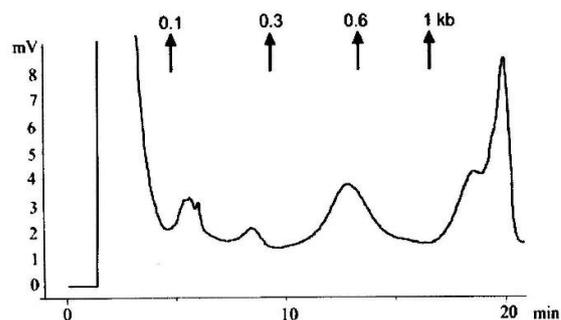


Figure 3. Analysis of second strand cDNA synthesis by IP RP HPLC. The double stranded cDNA (14 µl) was injected on to the DNASep® column and the DNA eluted after 18 min was fractionated and used in the construction of cDNA libraries enriched for larger inserts. The arrows above the chromatogram show the relative retention times for molecular weight markers under the same experimental conditions.

Other novel protocols including SMART-PCR and suppression subtractive hybridization (SSH)-PCR describe potentially less sensitive yet relatively easy and cost-effective alternatives for amplifying cDNA from sub-microgram levels of RNA (Hillmann et al., 2009). However, non-radioactive IP RP HPLC eliminates the use of radioactivity and therefore makes it a safe alternative to existing protocols.

In summary, non-radioactive IP RP HPLC is an easy to perform, safe, reliable and quantitative method which also provides a potential for RNA fractionation (2) prior to cDNA synthesis in order to further improve the quality of synthetic cDNA libraries enriched for “long” mRNAs.

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