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A Single-Step PCR For the Amplification of Highly Repetitive PolyQ Stretches

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Abstract

The expression, misfolding and aggregation of long-repetitive polyglutamine (polyQ) residues contribute to a number of neurodegenerative diseases where, protein-aggregates are formed, their degradation process is still not understood. Thus, to understand these diseases, new model systems are required. We exploited *Dictyostelium discoideum,* a non-mammalian system and successfully expressed varying polyQ repeat lengths, which could help delineate the pathways.

Traditionally, the (CAG)/(CAA) repeats (coding for polyQ) used in different organism are PCR amplified from disease-related genes like, in case of *E. coli*, human DRPLA (containing the different number of (CAG)/(CAA) repeats) cDNA was used as a template. To avoid the use of different templates for the synthesis of varying (CAG)/(CAA) repeats, we have made use of a single step PCR, using only one template for the synthesis of varying lengths of (CAG)/(CAA) repeats. The advantages of this technique are as below:

- A single step PCR amplification enables generation of variable polyQ repeats.
- This protocol reduces the time and consumption of raw materials for preparing varying lengths of (CAG)/(CAA) repeats.
- This may help identify the molecular mechanism(s) of pathogenesis of polyQ-repeat diseases and be a useful screen to identify potential therapeutic compounds in a non-mammalian model.

Keywords

Dictyostelium, PCR, polyQ, protein-aggregates

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Contributions: MJ performed most of the experiments. PB performed the microscopy and few cloning. BS and GK contributed in designing and writing of manuscript. All authors have seen and approved the final version of the manuscript

There is no conflict of interest.

INTRODUCTION

There is a known association between the lengths of polyQ repeats and the severity of diseases in most of the organisms studied (Lu and Murphy, 2014). The polyQ tracts get translated into aggregates and cause neuronal degeneration leading to the dysfunction of precise neuronal subpopulations (Stoyas and Spada, 2018). To investigate the influence of different tracts of the polyQ stretches on aggregation kinetics, we wanted to express, reporter constructs containing a

range of glutamine residues. Initially, to clone polyQ stretches, screening of adult human occipital cortex cDNA and fetal human brain cDNA library was prepared, using ^{32}P-labeled polyQ oligonucleotide, as the probe for the isolation of DRPLA (Dentatorubral-pallidoluysian atrophy) cDNA clone, which consist of a range of polyQ stretches (Onodera *et al.,* 1995). Polyglutamine repeat-GST fusion proteins were made by PCR from the human DRPLA cDNA (Onodera *et al.,* 1996).

This conventional method was time consuming and required templates with varying (CAG)/(CAA) repeats. Here, we made use of PCR-based amplification using primers with restriction enzyme sites for directional cloning into the desired vector. The expression of the fusion protein [polyQ-enhanced yellow fluorescent (eYFP)] was driven under the constitutive promoter, *actin15*. This helped visualize the protein aggregates formed and further be used for filter retardation analysis. Thus, using simple cloning procedures we could generate highly repeated DNA sequences of defined lengths.

The present work was undertaken to clone reporter constructs with defined numbers of polyQ repeats and to investigate the influence of varying lengths of polyQ stretches on aggregation kinetics. Here, we have exploited the PCR method that uses an unusual unique troubleshooting technique of mispriming, where non-specific amplification and failure of amplification of specific sequence cause multiple bands or a smear production. Mispriming caused due to the usage of non-specific primers and also due to few optimization parameters like annealing temperature, $MgCl_2$ concentration, pH, template purity and concentration (Puskas and Bottka, 1995). We have used this defect for the production of a range of (CAG)/(CAA) stretches. A single template containing 120Q (pB17S), a non-specific synthetic primer with restriction sites for directional cloning and a vector having a reporter gene was used. The non-specific primer anneals at multiple CAG/CAA positions on the template and results in the amplification of varying lengths of the amplicons having different repeat sequences coding for polyQ. We then selected the required range of (CAG)/(CAA) repeats and confirmed them by sequencing.

MATERIALS AND METHODS

Plasmid

Plasmid (pUAST attb used for fly cloning) was a kind gift from Prof. Namita Aggarwal (Department of Zoology, University of Delhi, India) that harbors a 120 (CAG)/(CAA) repeats plus the N-terminal human *huntingtin* region cloned in the polylinker between EcoRI,XhoI, where Xho1 site was not restored. We thus amplified the (CAG)/(CAA) repeats using the endogenous sequence of the vector. This amplicon having the 120Q repeats was directly cloned in the pB17S between SacI and BamHI sites. The plasmid (pB17S-120Q) obtained was transformed in competent bacterial cells (*E. coli* DH5α) and grown in Luria-Bertani (LB) broth (HiMedia, M575) supplemented with 100 µg/mL ampicillin (Merck, 171254). Cells were harvested before the cultures reached stationary phase (A_{600} of 0.7), condition important for maintaining the stability of repeats in *E. coli*.

To express the varying lengths of (CAG)/(CAA) repeats in *D. discoideum* we performed a single PCR using pB17S-120Q as a template. The primer combination used is shown below:

Primer	Restriction Enzyme	Oligonucleotide (5' to 3') (Restriction sites are underlined)	Tm	Length (bp)
FP	SacI	ATCAGAGCTCCTCAAAAGCTTCCAACAGCAACAG	70°C	34
RP	BamHI	TAGTGGATCC AGGTGGTGGCGGTTGTTGCTG	74°C	31

Table 1: The primer pair used for the amplification of varying polyQ repeat lengths. The restriction sites used for directional cloning are underlined.

The polyQ repeats were expressed under *actin 15* promoter as a fusion protein with eYFP at the C-terminal (pB17S). Vector (Fig. 1) consists of two antibiotic resistance gene, ampicillin (for selection in bacteria) and G418 (Geneticin, for selection in *Dictyostelium*).

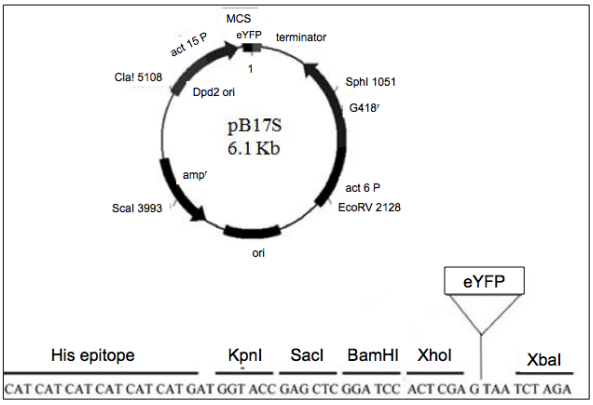

Fig 1: Schematic representation of the pB17S expression vector. The regions encoding ampicillin resistance (*amp*^{r}), the origin of replication for Dpd2 (Dpd2 ori) and the *E. coli* plasmid (ori), the constitutive *actin-15* promoter (*act15*), the neomycin resistance cassette (*Tn5 Neo*^{r}), the fluorescent protein coding region (*eYFP*) and the multiple cloning site (MCS) are indicated. Details of the MCS are shown in the lower panel.

PCR analysis

Prepare the PCR master mix for the amplification of (CAG)/(CAA) repeats using the following components:

Component	Stock Concentration	Working concentration	Reaction Volume (µL)
Phusion buffer (1.5 $MgCl_2$ mM) (F530S)	5x	1x	20
Template (pB17S-120Q vector)	100 ng/µL	100 ng	1
Forward Primer	10 µM	0.4 µM	5
Reverse Primer	10 µM	0.4 µM	5
dNTPs (3B Black Biotech India ltd, 3B154)	2.5 mM	200 µM	8
Phusion Enzyme (2U/µL, F530S)	2 units/µL	1 unit	0.5
DMSO (CAS # 67-68-5**)**			3
DDW (Sigma, W5402)			55
Total			100

Table 2: showing the master mix for the PCR amplification. The amplification parameters for gradient PCR was as follows: 3 min hot start at 98^{0}C; followed by 35 amplification cycle consisting of 30 sec cyclic hot start at 98^{0}C; annealing at 65^{0}C ± 10^{0}C for 45 sec; elongation at 72^{0}C for 15 sec and a final extension of 5 min at 72^{0}C.

Note 1: To achieve mispriming of reverse primer in PCR certain modifications were done. Magnesium concentration was kept low at 1.5 mM as magnesium ions stabilize base pairing. Since it is difficult to amplify GC rich regions due to its stability issues, DMSO, an organic additive was used that indirectly facilitates the annealing of primers to the template and enhances the amplification.

Agarose data analysis

- PCR products were analyzed on 1.2% agarose gel (A9536, Sigma) in 1x TAE electrophoresis running buffer with 0.5µg/mL EtBr.
- Results of the amplified products were visualized and photographed using Omega Lum™ G Imaging System. We observed a smear on the gel that was of varying stretches of (CAG)/(CAA) repeats as shown in Fig. 2A.

Extraction, digestion and ligation

- Smear bands less than 500 bp (as we needed polyQ repeats less than 120 glutamine repeats i.e. 360 bp) was extracted using Thermo-Scientific Gene JET, Gel Extraction Kit (K0691). The vector pB17S and the eluted products were double digested overnight with SacI and BamHI (both from Thermo-Scientific) at 37^{0}C.
- The digested vector (Fig. 2B) and smear bands (Fig. 2C) were purified using elution kit, quantified and ligated overnight at 16^{0}C in different ratios of vector and insert. They were incubated with 1 U of T4 DNA Ligase (EL0014, Thermo Scientific) in the supplied buffer.

Bacterial transformation and plasmid isolation

- 10 µL of the ligation mixture was added to 100 µL of competent *E. coli* cells (DH5α), transformed using $CaCl_2$ method (Chung *et al.,* 1989) and plated on LB-Amp plate followed by overnight incubation at 37^{0}C.
- Colonies were individually picked and inoculated in 2 mL LB-Amp media followed by overnight incubation under shaken conditions at 37 ^{0}C.
- Plasmids were isolated using protocol given in Sambrook *et al.,* 2006 and the positive transformants were verified using various restriction digestion analyses. The fall out of the varying (CAG)/(CAA) repeats having eYFP (700 bp) were verified using SacI and XbaI digestions, followed by 1% agarose gel electrophoresis as shown in Fig. 3.
- All plasmids with required (CAG)/(CAA) repeats were inoculated in 50 mL LB-Amp media for midi plasmid isolation as given in Sambrook *et al.,* 2006.

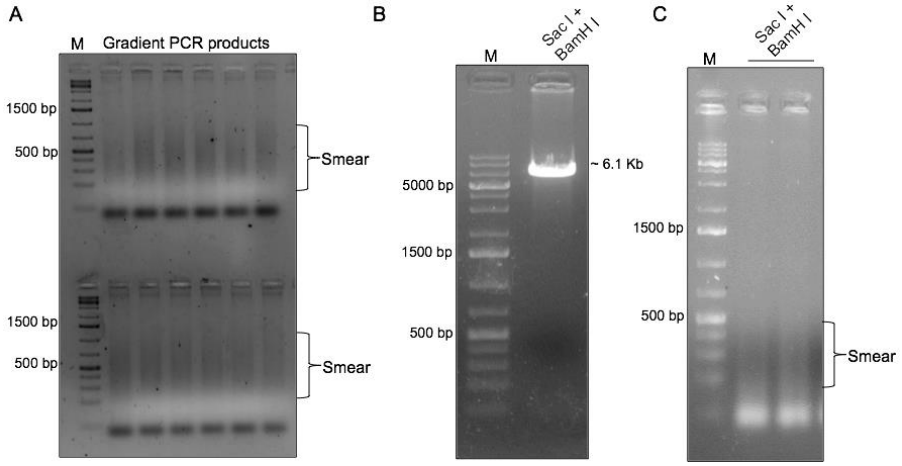

Fig 2: PCR amplification for varying (CAG)/(CAA) repeats. (A) Gradient PCR amplification using pB17S-120Q vector as a template with primer combination (mentioned in the text) was carried. A smear on the gel was observed. (B) The vector pB17S was double digested with SacI and BamHI and eluted. A band length of ~6.1 kb was observed. (C) The 500 bp and lower obtained in (A) was double digested (SacI and BamHI) and eluted. A smear of less than 500 bp can be observed. [M: DNA marker, 1kb DNA ladder, Thermo scientific].

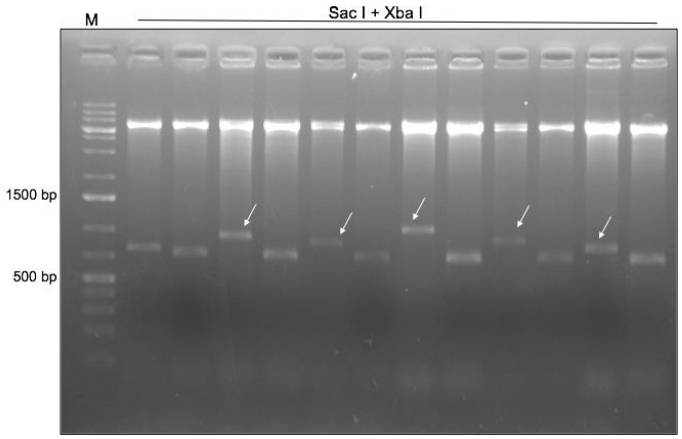

Fig. 3: One representative gel picture of the restricted (SacI and XbaI) plasmids obtained having varying (CAG)/(CAA) repeats with eYFP. The fallouts of different lengths marked with arrows can be observed. [M: DNA marker].

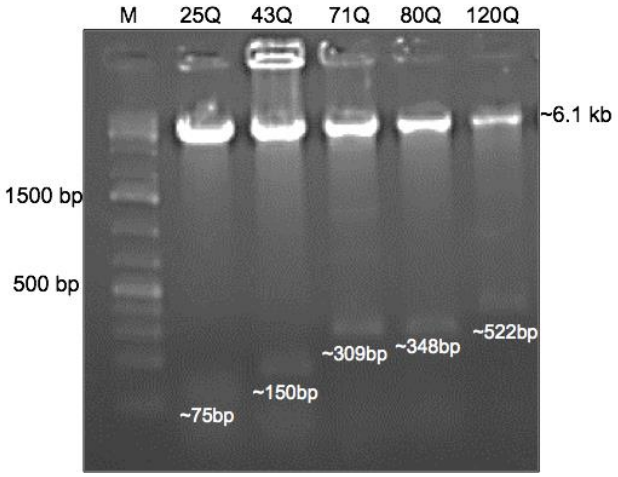

Fig 4: One representative gel picture for the confirmation of numbers of (CAG)/(CAA) repeats coding for varying polyQ stretches after digestion with SacI and BamHI is shown. The size of the different (CAG)/(CAA) repeats observed is marked that codes for different polyQ lengths. [M: DNA marker].

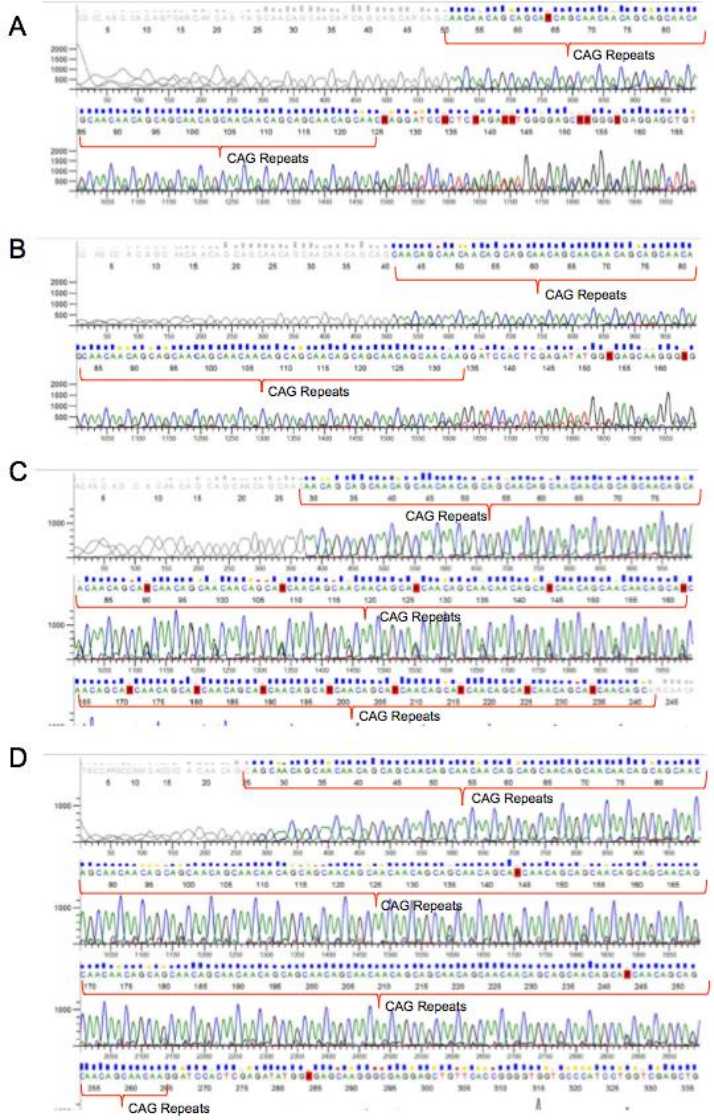

Fig 5: Electropherogram sequencing data of different (CAG)/(CAA) repeats coding for varying polyQ stretches that were expressed in *D. discoideum*. (A-D) shows sequences for 25, 43, 71, and 80 Q.

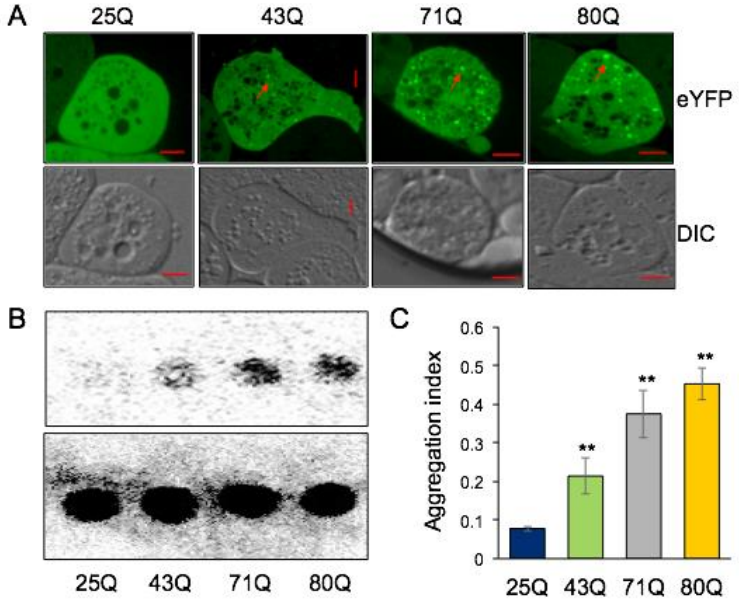

Fig 6: Analyses of polyQ aggregates. (A) Confocal images (top panel) showing polyQ aggregates (marked by red arrows). Lower panel are bright-field images of the same. (B) Filter retardation assay. Upper panel shows SDS-insoluble aggregates while the lower panel shows the total aggregates. (C) Aggregation index in varying polyQ stretches. [n= 3; *****p* **value; <0.01].**

RESULTS AND DISCUSSION

The present method for expressing varying lengths of polyQ in *D. discoideum* was very successful. We were able to express these fusion proteins having 25-120 polyQ stretches. A representative gel of final digestion with SacI and BamHI separated on 1.5% agarose gel is shown in Fig. 4. The numbers of (CAG)/(CAA) repeats were confirmed after sequencing (Fig. 5) from Scigenom Pvt. Ltd, India. Sequencing confirmed the result that these smear bands consists of a range of (CAG)/(CAA) repeats that code for 25Q, 43Q, 71Q and 80Q stretches.

Next, we wanted to confirm if the fusion proteins were expressed in *D. discoideum* formed protein aggregates. The constructs were transformed into *D. discoideum,* Ax2 cells by electroporation method (Sharma *et al*., 2018) and the transformants were selected on the antibiotic G418. They were sequentially selected and grown maximally with 100 µg/mL of G418. The eYFP allowed us to visualize the protein aggregates as fluorescent dots. Representative confocal images (Ex=488 nm; Em=545 nm) are shown in Fig. 6A where red colored arrows mark the aggregates.

This was further analyzed using filter retardation. Here, the prepared cell lysate was divided into two aliquots where, one aliquot was absorbed on a 0.2 µm nitrocellulose membrane (MDI SCNJ, India) to evaluate the total amount of polyQ proteins, whereas the other aliquot was absorbed on nitrocellulose membrane soaked on 2% (w/v) SDS to arrest the polyQ aggregates. The amount of polyQ aggregates retarded on the membrane due to insolubility in SDS was quantified using anti-GFP antibody (Sigma, G1544) by immunoblotting (Fig. 6B). The aggregation index (polyQ aggregates over the total polyQ proteins) was plotted (Fig. 6C). We could observe increasing aggregation index with increasing lengths of polyQ stretches.

CONCLUSIONS

Here we describe a simple approach for generating (CAG)/(CAA) repeats that involves a single-step PCR amplification followed by standard cloning protocol. Earlier methodologies used for such amplifications required multiple cloning strategies. The method presented here is both cheap, fast and can be easily adapted to produce any desired (CAG)/(CAA) repeats for further studies.

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