Ultrafiltration-based in vitro assay for determining polyamine binding to proteins

Palanimurugan R (pmurugan@gmail.com)
Biocenter, Institute for Genetics, University of Cologne, Zülpicher Str. 47a, D-50674, Cologne, Germany
R Jürgen Dohmen
Biocenter, Institute for Genetics, University of Cologne, Zülpicher Str. 47a, D-50674, Cologne, Germany

Method Article

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Abstract

We have established an ultrafiltration-based _in vitro_ assay for determining polyamine binding to proteins. In the protocol described here we have used _Saccharomyces_ _cerevisiae_ ODC antizyme protein produced in _E. coli_ and radioactive polyamines to measure the direct binding of polyamines to ODC antizyme protein.

Introduction

Polyamines are small aliphatic polycations present in all cells. Polyamines are known to have several important functions, which are essential for life \(Ref. 1). The cellular polyamine pool contains several modified forms of polyamines, however putrescine, spemidine and spermine are the most abundant among others. Normally polyamines exert their molecular functions by direct binding to cellular macromolecules such as DNA, RNA and proteins \(Refs. 1 and 2). We have established an in vitro polyamine binding assay that enables identification and characterization of novel polyamine targets. Using this ultrafiltration-based protocol, we have assayed polyamine \(spermidine or spermine) binding to yeast or human ornithine decarboxylase \(ODC) antizyme \(AZ) \(Refs. 3 and 4) protein produced in _Escherichia coli_. Apart from being easy and fast, this protocol requires only small quantities of purified proteins for determination of polyamine binding. Combined with protein fractionation methods, this protocol can be applied for the identification of novel intra or extra cellular polyamine binding proteins. The protocol given below describes determination of polyamine binding for 6His-tagged _S. cerevisiae_ ODC antizyme \(OAZ1). An adopted version of this protocol was used to determine polyamine binding to human antizyme fused to maltose binding protein \(Ref. 4).

Reagents

1. Buffer \(50 mM Tris-HCl, pH 7.8 at 4°C) 2. \[3H]-spemidine \(PerkinElmer, cat No: NET522001MC) 3. \[14C]-spermine \(GE Healthcare, cat No: CFA511) 4. 6His-OAZ1 \(_S. cerevisiae_) in IMAC elution buffer \(50 mM Tris-HCl, pH 7.8 containing 250 mM imidazole) after expression in _E. coli_ and purification by immobilized metal affinity chromatography \(IMAC). 5. Mock preparation from _E. coli_ cells transformed with an empty vector not expressing 6His-OAZ1. 6. Scintillation liquid \(picard) 7. Microcentrifuge tubes \(Eppendorf)

Equipment

1. Refrigerated Microcentrifuge 2. Centrifugal filter, Modified polyethersulfone \(PES) 10K \(VWR, Cat No: 82031-350) 3. Scintillation counter

Procedure

1. Keep all reagents on ice. Pre-cool the centrifuge to 4°C. 2. Label the microcentrifuge tubes and keep them on ice. 3. Prepare the polyamine binding mix in the pre-cooled microcentrifuge tubes as shown Table A & B shown in figures section. 4. Mix the polyamine binding mix gently and incubate on ice for 60 min. 5. Place the filtration device in a 1.5 ml collection tube and pre-cool on ice. 6. After the 60 min incubation time, transfer 100 µL of the polyamine binding mix to the appropriately labelled filtration devices. 7. Filter the free polyamines by spinning at 2500xg for 5 min at 4°C 8. Prepare microcentrifugefuge tubes with 1 mL scintillation liquid \(two tubes with scintillation liquid for every filter device used) and keep them at room temperature. 9. Remove 10 µL of the retentate from inside the cut-off filter device and add it to microcentrifuge tube with scintillation liquid marked as “retentate”. 10. Remove 10 µL of filtrate from the collection tube of the filtration device and add it to the second microcentrifuge tube with scintillation liquid marked as “filtrate”. 11. Vortex for 10s and proceed with scintillation counting. 12. From the resulting CPM \(Counts per Minute) values, calculate the percentage of protein-bound polyamines using the following formula. Percentage polyamine Binding = \{\ (CPMretentate- CPMfiltrate)/ CPMretentate} x 100

Timing

About 2 hours

Troubleshooting

No Binding: Verify that the protein did not precipitate during the assay. Perform the polyamine binding assay immediately after purification of protein. Strictly avoid freezing and thawing of purified OAZ1.

Anticipated Results

1. Binding: CPM in filtrate will be less than retentate 2. No Binding: CPM in filtrate will be similar to retentate Representative results for spermine and spermidine binding to _S. cerevisiae_ OAZ1 are shown in Fig. 4 and Supplementary Fig. 9 of Kurian et al. Nature, 2011, Doi: 10.1038/nature10393.

References

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Figures

Table A. ^{3}H-spermidine binding mix:

Reagents	Buffer	Mock	OAZ1	Control Proteins
Mock	-	X µL	-	-
OAZ1 (10 µM final)	-	-	X µL	-
Control Protein	-	-	-	X µL
^{3}H-spermidine (10 µM final)	15.30 µL	15.30 µL	15.30 µL	15.30 µL
IMAC elution buffer	84.70 µL	(84.70-X) µL	(84.70-X) µL	(84.70-X) µL
Total	100 µL	100 µL	100 µL	100 µL

Table B. ^{14}C-spermine binding mix:

Reagents	Buffer	Mock	OAZ1	Control Proteins
Mock	-	X µL	-	-
OAZ1 (10 µM final)	-	-	X µL	-
Control Protein	-	-	-	X µL
^{14}C-spermine (10 µM final)	2.3 µL	2.3 µL	2.3 µL	2.3 µL
IMAC elution buffer	97.7 µL	(97.7-X) µL	(97.7-X) µL	(97.7-X) µL
Total	100 µL	100 µL	100 µL	100 µL

Figure 1

Table A & B Radio-labeled polyamine binding mix