European Science Methodical Journal
ISSN (E): 2938-3641
Volume 2, Issue 5, May - 2024

OPTIMIZATION OF BINDING BUFFER ION FOR COMBINATORIAL SELECTION OF SSDNA APTAMERS FOR FLAVONOIDS

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Abstract:

Aptamers are synthetic, single stranded oligonucleotides that bind with high affinity and specificity to molecular targets. Binding buffer, is an influential component in the combinatorial selection of an aptamer for effective interaction between aptamers and their target. This paper optimized the buffer composition requisite for the development of flavonoid aptamer using combinatorial chemistry procedure called systematic evolution of ligands by exponential enrichment technology (SELEX) and luteolin as a model. Influence of mono and divalent cations together with the ionic strength alteration via binding reaction between aptamer and luteolin under various buffer conditions were determined. Sodium was found to be more efficient ion in binding buffer than potassium. In the case of divalent ions, although magnesium revealed insignificant effect on binding affinity, it played a vital role in the binding stability. Furthermore, alteration in the ionic strength did not enhance the binding affinity. Sodium phosphate buffer was demonstrated to be effective for in vitro selection of ssDNA aptamer for luteolin and might be suitable for other flavonoids. It was established that by minimizing the buffer requirement towards the development of aptamer for flavonoids the cost and time consumption could be reduced considerably.

Keyword: Binding buffer; aptamer; SELEX; flavonoids.

Introduction

Aptamers such as ribonucleic acids (RNA) and single-stranded deoxyribonucleic acid (ssDNA) are oligonucleotides which can bind to the targets with high affinity and specificity depending on their particular three-dimensional structures [1]. Over the years, aptamers have been intensively used to detect sundry targets including proteins, drugs, cofactors and antibodies [2]. Primarily, aptamers have been selected via the protocol of combinatorial chemistry popularly known as systematic evolution of ligands by exponential enrichment technology (SELEX) [3]. Selections of aptamer are distinguished by the reiteration of successive target binding step and unbound oligonucleotides expulsion followed by elution, amplification, and purification of the

selected ssDNA molecules [4]. They remain prospective towards molecular detection in analytical systems in terms of recognition, purification or separation of target molecules [5]. Although aptamers are specific to their target, the buffer system play an important role for their specificity to the similar structure target. Furthermore, even though aptamers and their buffer system has been extensively studied with a biomolecule such as protein and xenobiotic compounds, very little has been explored for flavonoids and its buffer system.

Flavonoids is a biologically active compounds of plant secondary metabolites often constitute a substantial part of the human diet [6]. Lately, flavonoids became a focal point in nutraceutical industry due to their diverse pharmacological benefits concerning anti-bacterial, anti-allergic, anti-inflammatory and anti-thrombotic activities [7]. Indeed, flavonoids behave as antioxidants wherein they can directly trap reactive oxygen species, chelate transition metals involved in radical formation, inhibit enzymes that are responsible for producing superoxide anions and prevent the peroxidation process by reducing peroxyl and alkoxyl radicals [8]. Considering such notable benefits, flavonoids have been selected as target for this study.

Screening is essential to identify the conditions for achieving desired results once an appropriate biological macromolecular sample is chosen. Such screening process must be accompanied with the choice of optimum binding buffer for *in vitro* selection of an aptamer. This optimization is mandatory because of the phosphate group present in the backbone of DNA molecules contained negative charges. Consequently, the occurrence of charge repulsion impedes the binding with the target in the absence of partner. In the living cell, positive charges from histone protein can neutralize only half of such negative charges while the positive ions of Mg^{+2}, Ca^{+2}, Na^{+} and K^{+} neutralizes the rest of the negative charges. Therefore, inclusion of partners is indispensable to improve the target-specific ssDNA aptamers binding. In this view, various researchers incorporated monovalent and divalent ions including Na^{+}, K^{+}, Mg^{+2} and Ca^{+} into the binding buffer to enhance the ssDNA folding in a particular three-dimensional structure prior to the binding with the target molecules and binding stability [9, 10]. Researches revealed that the presence of monovalent and divalent cations such as Na^{+} and Mg^{2+} can block the negative charge repulsion to allow the DNA backbone to regulate stronger folding and interact indirectly with the target [11]. It was also reported that high concentration of monovalent cations could prevent some nonspecific binding to the target [12]. Conversely, higher concentrations of divalent cations such as Mg^{2+} were shown to facilitate the formation of secondary structures and thereby could enhance the non-specific binding [13].

Earlier, the ssDNA aptamer designed as LUT#3 that bound to luteolin with high affinity and specificity with K_d =131 nM was exploited from random library of 10^{15} ssDNA oligonucleotides after eight iterations using the binding buffer composed of 100 mM of NaCl, 20 mM of Tris-HCl (pH 7.6), 2 mM of $MgCl_2$, 5 mM of KCl, 1 mM of $CaCl_2$ and 0.02% of Tween 20 [14]. Figure 1 displays the secondary structure of LUT#3. Despite high binding affinity of the achieved buffer the preparation method was expensive and time consuming. Driven by this idea, we optimized the binding buffer conditions for *in vitro* combinatorial selection of specific aptamers for luteolin.

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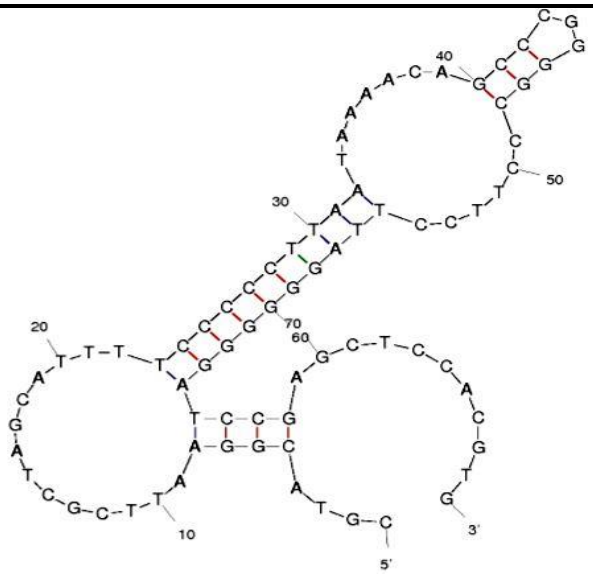

Figure 1: Secondary structure model of LUT#3 aptamer as predicted by M fold tool (Zuker, 2003).

MATERIALS AND METHODS

Materials and binding buffers

All the buffers were prepared using ultra-pure water (direct Q from Millipore). Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich (USA). Sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate were procured from QRëC (Selangor, Malaysia). Magnesium chloride, potassium chloride, sodium hydroxide, sodium chloride, calcium chloride, urea, and TE buffer were obtained from Biobasic Inc. (Ontario, Canada). The chemical composition of prepared buffers used in the experiment were summarized in Table 1**.**

Table 1. Chemical composition of various buffers used in the experiment**.**

Buffer	Composition & pH
Sodium phosphate	10 M Na_2HPO_4/NaH_2PO_4 and 2 mM $MgCl_2$, pH 7.4
Potassium phosphate	10 M K_2HPO_4/KH_2PO_4 and 2 mM $MgCl_2$, pH 7.4
Phosphate (PBS)	2 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4
Phosphate plus NaCl	2 mM KH_2PO_4, 10 mM Na_2HPO_4, 2M NaCl, pH 7.4
Phosphate plus KCl	2 mM KH_2PO_4,10 mM Na_2HPO_4,100 mM KCl, pH 7.4

Preparation of LUT#3 aptamer

LUT#3 cloned aptamer in PSTBlue-1 vector from E.coli JM109 were obtained from library stock in Nutritional Biochemistry Laboratory, Faculty of Biosciences and Medical Engineering,

Universiti Teknologi Malaysia. The clones were cultured on LB agar plate and the colonies were left to grow overnight at 37℃. The amplification of LUT#3 aptamer was conducted by colony PCR. The colonies were inoculated with pipette tips and re-suspended in individual PCR tubes containing the PCR mixture. The PCR tubes were placed in an Eppendorf master cycler theromcycler and the amplification conditions were 10 min at 94 ℃ (initial cell breakage and DNA denaturation), 35 cycles of 1 min at 94 ℃, 1 min at 47 ℃, 1 min at 72 ℃, and then 10 min at 72 ℃ after the last cycle. In order to separate dsDNA PCR product into the proper ssDNA strand after the amplification step, denaturing PAGE containing 12% acrylamide was performed. The desired DNA bands were cut out and placed in a 1.5 mL microcentrifuge tube. Then, 300 μL of Crush and Soak Solution (500 mM of NH4OAc, 0.1 mM of EDTA, 0.1% of SDS) were added and the ssDNA fragments were recovered from the gel after incubation at 70 ℃ for 2 h with gentle shaking. The recovered ssDNA concentration was quantified by ND-1000 NanoDrop and used as initial ssDNA for the next experiment.

Determination of metal cations and its ionic strength effect on aptamer binding

The impact of metal cations ionic strength on the binding affinity of the luteolin-specific aptamer (LUT#3) was determined by allowing it to react with luteolin-coated magnetic beads under varied binding buffer conditions. Following the FluMag-SELEX method [5], the *in vitro* selection was performed via binding, washing and elution steps amid multiple buffer conditions as summarized in Table 1. Ionic strengths were varied by increasing the concentration of cations in phosphate buffer.

In each experiment, the luteolin-coated beads were washed eight times in 500 µL of binding buffer (BB) and re-suspended in 100 µL of BB. 0.5 µg LUT#3 aptamer was added to 400 µL of BB. The ssDNA pool was unfolded (denatured) in the binding buffer for a period of 10 min at 90 ℃ before being plunge in ice for 15 min. Next, the samples were incubated at room temperature (RT) for 5 min prior to the exposure of binding buffer. Finally, the 400 µL of aptamers solution was added to the 100 µL of luteolin-coated beads suspension and incubated at RT for 60 min accompanied by gentle rotation and tilting. The unbound oligonucleotides was removed by repeated washing with 500 µL of BB for 5 times. The luteolin-bound aptamers were eluted by heating the beads at 80 ℃ for 10 min with 200 µL of elution buffer (EB) comprised of 40 mM Tris–HCl, 10 mM EDTA, 3.5 M of urea, 0.02% of Tween 20 (pH 8.0) accompanied by shaking. This step was repeated four times in order to recover all traces of bound ssDNA. The eluted ssDNA aptamer from the luteolin-coated beads were subjected to precipitation using ethanol before being re-suspended in 10 µL of TE buffer. In each experiment, the relative affinities of the developed aptamers to luteolin were evaluated by measuring the eluted ssDNA oligonucleotides (bound DNA). The ND-1000 NanoDrop was used to quantify the percentage of binding at each buffer condition.

RESULTS AND DISCUSSION

The presence of monovalent and divalent cations in the binding buffer is responsible for maintaining the constant ionic strength. As aforementioned, the positive ions are attracted towards the negative charge of nucleic acid resulted from the surrounding ions sheath formation

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(ion atmosphere). High mobility of this dense ionic environment containing with varying conditions (Figure 2) makes the description complex [15]. The binding of ions such as Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} to DNA being mostly electrostatic in nature displays weak and contradictory DNA selectivity [16]. Besides, the results of competitive experiments exhibit that the amount of absorbed Na^{+}/K^{+} on DNA have weaker selectivity for Na^{+} than K^{+} at various conditions [17]. Generally, the competition between similar ionic species (K^{+} with Na^{+} or Ca^{2+} with Mg^{2+}) is only qualitative wherein such competition is much more sensitive to the molecular details of the DNA-ion-solvent interaction. The schematic ion atmosphere in Figure 2(i-iv) depicts several experimentally observed trends. Figure 2(i) shows the increase in coion (negative binding anions) depletion compared to counterion accumulation due to higher total ionic concentration. 2(ii) shows the dominance of divalent cations in the atmosphere. Figure 2(iii) shows the domination of monovalent cations in the atmosphere that leads to a tighter spatial association of the ions around the nucleic acid. Figure 2(*iv*) displays approximately equal concentrations of monovalent and divalent cations. In the presence of excess monovalent ions, some divalent ions are expected to remain close to the nucleic acid [15].

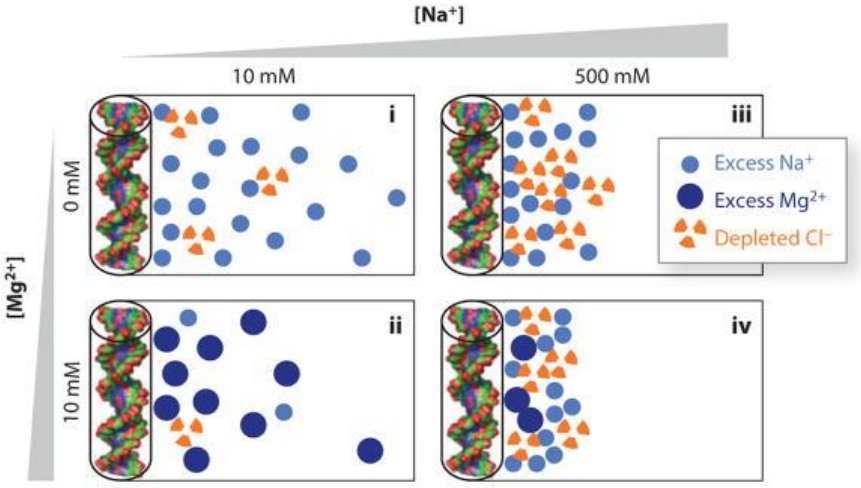

Figure 2: Schematic diagram representing the ionic atmosphere around DNA at different solution conditions (circles are excess ions and triangles are depleted ions) [15].

Preparation of LUT#3 aptamer

The LUT#3 cloned aptamer from library stock were cultured on LB agar plate and then amplified by colony PCR. It was imperative to isolate double-stranded DNA (dsDNA) to the proper ssDNA after the PCR amplification step to be used as initial DNA pool for the next experiment. Denaturing PAGE containing 12% of acrylamide and 7 M of urea in TBE buffer were applied to segregate the aptamers from their complement. The desired DNA fragments (fluorescent bands) were verified with the help of an UV transilluminator as depicted in Figure 3. The fluorescent bands represent the isolated LUT#3 aptamer and other bands represent the DNA complement. The desired ssDNA fragments were cut out and crushed from the gel after the incubation at 37 ℃ for 12 h in crush and soak solution. Later, the solution was centrifuged

at maximum speed, the supernatant were collected and subjected to ethanol precipitation. The recovered ssDNA concentration and purity were quantified by ND-1000 NanoDrop and used as initial ssDNA for the next experiment.

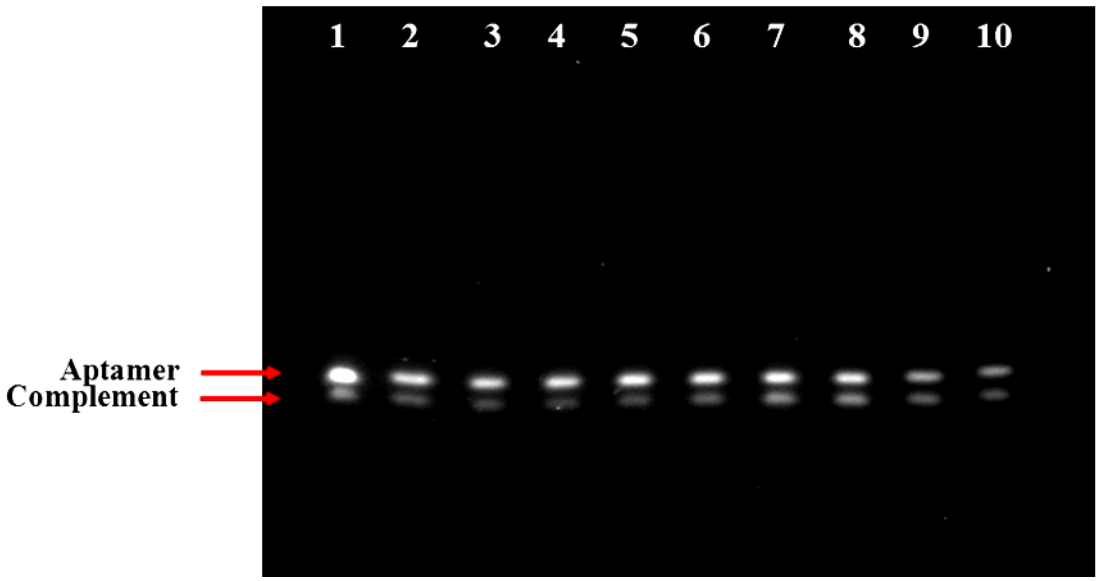

Figure 3: Urea / denaturing PAGE. The PCR product was dispersed in lanes (1-10). The fluorescent bands represent the isolated ssDNA aptamer and other bands represent the DNA complement.

Effect of mono and divalent cations on aptamer binding affinity

Additional rounds of *in vitro* selection at multiple buffer conditions were performed to determine the effects on binding affinity. Figure 4 depicts the buffer nature dependent binding ability of LUT#3 aptamer. The LUT#3 aptamer exhibited utmost binding affinity in buffers supplemented with Na^+, where the achieved affinity for the sodium phosphate and phosphate buffer (PBS) was 74.33% and 68.48%, respectively. Conversely, identical buffer supplemented with K^+ revealed weak binding affinity with 15.33%. It is suggested the role of Na^+ cations in blocking the negative charge of DNA and permitting the DNA backbone to regulate stronger folding was clearly manifested. This observation may be due to the different interaction of Na^+ and K^+ with DNA due to their difference in the ionic radii. It is believed, K^+ ions preferred in binding to the grooves of DNA while Na^+ ions was resided near the phosphate groups outside the DNA. Furthermore, K^+ ions favored the direct binding to the electronegative sites of the DNA bases and to the O4* atom of pentose whereas for Na^+ ions the major site for direct binding were O1P atom of the phosphate group [18].

Regardless of the existence of Mg^{+2} in both sodium and potassium phosphate buffers they disclosed different binding affinity, which indicated ineffectiveness of Mg^{+2} without any direct influence on binding. Moreover, it was acknowledged that Mg^{+2} could stabilize the secondary and tertiary structure of aptamer [19]. Aptamers that showed most excellent binding affinity were less dependent on Mg^{+2} ions than the one with weaker binding [13]. Thus, *in vitro*

selection of aptamers in the presence of 1 to 2.5 mM of Mg^{2+} could enhance their inflexibility and produce high binding affinity [11]. Complete removal of potassium and calcium did not affect the binding affinity between luteolin and aptamer in the presence of Na^{+} and Mg^{+2} cations. This indicated that K^{+} and Ca^{+2} had insignificant influence on the interaction among luteolin and the DNA aptamer. This observation supported the fact that both Na^{+} and Mg^{+2} cations could effectively occupy an area up to 10 Å from the DNA duplex surface as well as the distribution of Mg^{+2} ions around an isolated DNA duplex was more compact than that of Na^{+} ions [11]. In short, LUT#3 aptamer revealed excellent binding affinity for buffers supplemented with Na^{+} and weak binding affinity for bufferd supplemented with K^{+}.

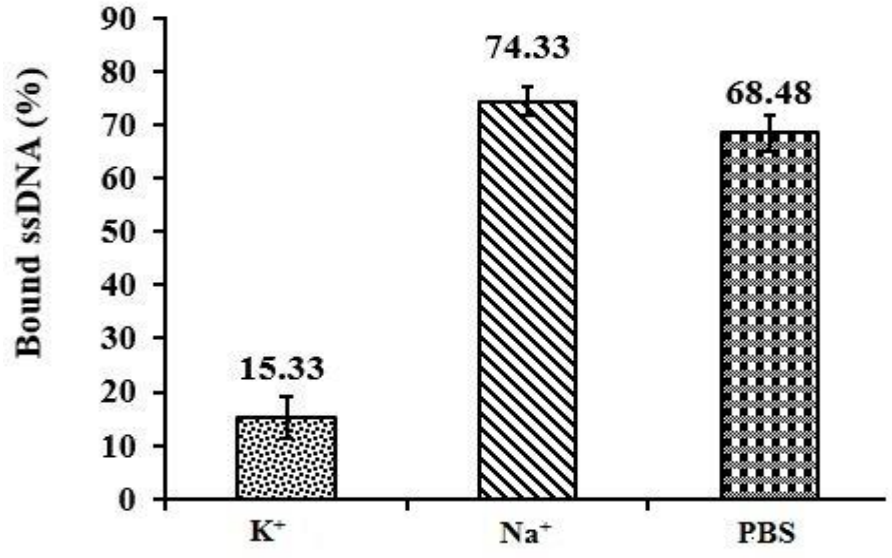

Figure 4: Na^{+} or K^{+} cations dependent binding capacity of LUT#3 aptamer (data represent mean ± standard deviation. n=3).

Effect of ionic strength on aptamer binding affinity

It was reported that an improvement in the ionic strength could shield the electrostatic interaction between DNAs and their targets [20]. Electrostatic interaction played a vital role towards the binding of aptamers with small molecular targets [21, 22]. For in-depth understanding on the mechanism of aptamer binding affinity, the binding of LUT#3 to luteolin-coated beads was tested in phosphate buffer plus 2M of NaCl or 100 mM of KCl. Figure 5 shows the ionic strength dependent binding capacity of LUT#3 to luteolin-coated beads. The binding capacity was not affected noticeably due to the inclusion of 2M of NaCl or 100 mM of KCl into the reaction mixture. Meanwhile, the observed slight reduction in the binding capacity (51.25% for PBS+KCl and 61.49% for PBS+NaCl) was primarily ascribed to the absence of

Mg^{+2} in binding buffer. Based on these results, it was suggested that the electrostatic interaction played insignificant role in the binding of aptamers to luteolin as flavonoids. It is well known that π-π stacking and hydrogen binding interactions usually contribute to the specific interaction between aptamers and aromatic ligand [11]. Such binding involves the insertion of a planar fused aromatic ring system between the DNA bases and ligands, leading to p-electron overlap. In the present case, it is suggested this type of binding was stabilized by the stacking interactions and thereby remained less sensitive to the ionic strength [23]. However, flavonoids structure having 3 carbon units in the form of an oxygenated heterocyclin ring (C ring) could participate in the π-π stacking and hydrogen binding interaction with the aptamers.

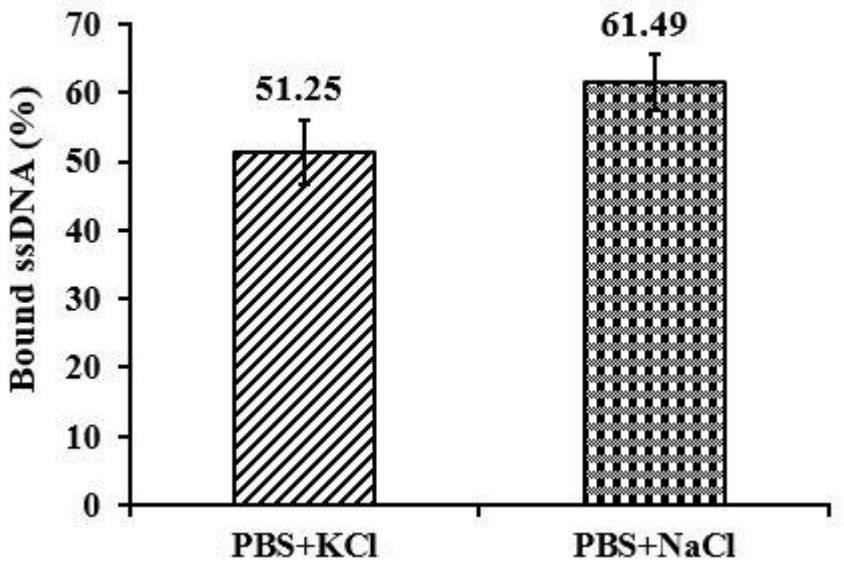

Figure 5: Ionic strength dependent binding capacity of LUT#3 to luteolin-coated beads (data represent mean ± standard deviation. n=3).

CONCLUSION

This study clearly showed that both mono and divalent cations play an important role in the compensation for the negative charge of oligonucleotides and stability of its structures. Specifically, monovalent sodium was found to be more efficient than monovalent potassium in luteolin specific aptamer binding for luteolin. The binding efficiency can be improved and the consumption of reagents can be controlled by optimizing binding buffer composition.

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