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REVIEW ARTICLE

The minimum aptamer publication standards (MAPS guidelines) for *de novo* aptamer selection

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ABSTRACT

Aptamers were first described in 1990 and since then many aptamers have been reported in the literature for numerous applications in both diagnostics and therapeutics. However, as with most fields, missing or unclear information presented in the publication makes it difficult to replicate some of the work described in the literature. To increase the reproducibility of the data and facilitate academic laboratories and industrial companies to develop reliable aptamer work, essential guidelines should be proposed and followed in any aptamer publication, especially in those that highlight *de novo* aptamer sequences. Here, we provide suggestions for authors, reviewers, and editors to follow when performing and reporting their aptamer work to ensure that we meet the minimum standards for publication of future aptamer sequences.

KEYWORDS: aptamers, guidelines, *in vitro* selection, minimum standards, reproducibility, SELEX

INTRODUCTION

It is now 30 years since the first papers describing aptamers were published (Ellington and Szostak 1990, Tuerk and Gold, 1990). In these past three decades, there have been several thousand aptamers generated and described in the literature. Targets range from metal ions (Hg^{2+}, As^{3+} and Cd^{2+}, Cu^{2+}, *etc.*) (Guo et al, 2021), very small molecules, such as glucose (Yang et al, 2014) and cocaine (Stojanovic et al, 2000), proteins and peptides (Shigdar et al, 2011), to whole organisms such as the parasite *Trypanosoma brucei* (Homann and Göringer, 1999). As well, there have been many adaptations and modifications to the traditional selection process, the Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Zhang et al, 2019). Finally, there have been advancements in technology to ensure that the measurement of specificity and affinity of potential aptamer sequences can be determined very precisely (McKeague et al, 2015). Despite these changes, the basic premise of aptamer development and applications remains the same (Freedman and Inglese, 2014).

As in the case of antibody or RNAi technologies, aptamers underwent an initial moment of euphoria and success that included a clinically-approved aptamer formulation (Pegaptanib/Macugen) (Ruckman et al, 1998; Ng et al, 2006). However, the acceptance of aptamers as affinity reagents that have their own unique set of advantages has been emerging in a steady albeit incremental manner over the last three decades. One of the limitations in these authors' experience is the lack of reproducibility of published data, in part due to the absence of standardised protocols that can critically determine specificity and affinity of aptamer binding. Given that the most pervasive reason for a general lack of reproducibility in scientific research is an incomplete protocol (Freedman and Inglese, 2014), the Aptamer Consortium, which is part of the International Society on Aptamers (INSOAP), felt it was timely to suggest best practice standards to meet when characterising and publishing new aptamer sequences. This paper will highlight pertinent information that should be reported regarding aptamer selection, characterisation, and application. First, similar to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) presented in 2009 (Bustin et al, 2009) and now are well accepted and required by journals for publication, we present the MAPS guidelines: the **m**inimum **a**ptamer **p**ublication **s**tandards for *de novo* aptamer selection (Table 3).

We also provide examples of ideal information and useful assays that will drastically help push aptamer applications forward, but that may not be required for a first selection publication. Together, we hope to prevent repeatability issues faced to date by aptamers (Bottari et al, 2020; Tao et al, 2020) and help ensure that aptamers do not meet the same ‘irreproducibility’ fate (Zong and Liu, 2019) suffered by antibodies (Baker, 2016).

APTAMER SELECTION

Besides naturally occurring aptamers like riboswitches, nearly all newly reported aptamer sequences are discovered using the traditional SELEX process or its variants (Sharma et al, 2017). Within this process, there are multiple parameters that may impact the discovery of high affinity aptamers as well as their final function. The critical parameters include choice of nucleic acid as the initial library, the length of the random region, the inclusion of primer binding sites, their specific sequences and complements to these sites, folding conditions for the selection library, metal cation concentration, buffer and pH, target immobilisation strategy and concentration, temperature, use of competitors/blocking agents, number of PCR cycles in each selection cycle, polymerase used for amplification, separation of double-stranded products (for DNA libraries), conditions for the *in vitro* transcription/purification (for RNA libraries) incubation time and temperature with target, molar ratio of nucleic acid to target in each cycle where possible, and partitioning conditions. In the case of cell-SELEX, the cell condition in culture is critical for successful aptamer selection. Stable performance of living cells, in terms of proliferative and morphological features, should be constantly verified over the entire selection process. In addition, cell cultures should be tested for contamination by mycoplasmas.

These conditions are generally optimised in each individual laboratory over time and then become the standard method. As such, some published papers will usually refer to previous papers for the selection criteria chosen. However, this information may be incomplete, or there may have been additional changes to the protocol over time that have not been published. We would therefore recommend that selection conditions are included in a table in the methods section or in supplementary information. Below, we provide a suggested format for reporting all relevant SELEX information in Table 1 and Table 2.

Table 1. Sample table for reporting all relevant selection conditions. Note: this information could be presented in an easy-to download supporting “excel” file.

Selection condition	Example of information
Library information	• Length of random region, primer binding sites and availability • Nucleic acid backbone and modifications • Size (nmols) and concentration of starting library • How was the library synthesised? Was the library PCR amplified prior to Round 1? • Was the library sequenced or otherwise characterised?
Folding conditions	• Temperature and time for each step
Buffer and pH	• Which buffer and pH was used?
Additional constituents of binding/ selection buffer	• Was any other ingredient added? • What were the storage conditions of the buffer and components (*e.g.*, made immediately prior to use, stored in freezer, could be stored in fridge for one week?)
Constituents of blocking buffer to reduce non-specific binding sites	• Were blocking agents or competitors used- and how?
Immobilisation of target	• Conditions of immobilisation; type of immobilisation and linker; how was immobilisation verified? • Concentration of target used for immobilisation.
Partitioning conditions	• What method was used to partition target bound from free aptamer? • What were the buffer conditions? • What were the incubation time and temperature
Negative/counter selection	• Was negative selection used? What type of negative selection? When was it used?
Preparation of Pool for each round	• Was there a single-stranded oligo generation step? • How was the library/pool quantified each round? • What are the details of oligonucleotide purification?
PCR conditions	• What PCR polymerase was used? • What were the PCR buffer conditions? • What were the primers • What were the PCR cycle parameters • How many PCR cycles? • How much sample was amplified? • How was the amplified sample analysed?

Table 2. Selection conditions for each round of a new SELEX experiment.

Round	Concentration of library to target ratio (when possible) or amount of target used	Volume of binding buffer	Temperature and length of incubation	Number and length of washes	Number of PCR cycles to amplify bound species	Cell density/ Conditions (Cell-SELEX)
Round 1	pmol:pmol and volume	x µl	x °C and x mins	x washes for x mins each	x cycles	1 x 10^x cells in adherent/ non-adherent conditions

SEQUENCE INFORMATION

Following selection, candidate aptamer sequences are identified through sequencing. Some laboratory groups continue to use traditional cloning and Sanger sequencing to identify sequences selected against the target. In this case, as many sequences as possible should be obtained to attempt to find "enriched" motifs. The choice of the final selection round(s) used for cloning and sequencing, and the decision to stop doing additional affinity selections, should also be briefly justified. For example, the final round of selection might not show the best binding to target (Schütze et al, 2011) When appropriate, binding assay results for selected rounds could be presented to indicate which selection round was chosen and this data should be presented as part of the results. It would be useful to acknowledge that the decision to stop doing additional rounds of selection often involves some degree of judgment that sufficient enrichment for desired functional properties has been achieved. In this context, it is useful to simply state the reason for the decision.

In the past ten years, there has been a general trend towards Next Generation Sequencing (NGS). NGS is a powerful method that provides millions of sequences from selection rounds. While protocols have developed particularly well over the years to make sense of this information, it is imperative that the method for choosing sequences for further interrogation is detailed (Komarova et al, 2020). For example, which NGS platform was used, which rounds were sequenced, what process and software(s) was used to analyse the raw data, how were the sequences clustered, what software was used for secondary structure prediction. Ideally, when possible, a representative list of sequences from the final affinity-enriched pools should be presented in the supplementary files and carefully checked for accuracy (Miller et al, 2021). Finally, when and where feasible, all raw sequencing underlying data should be deposited to an appropriate public repository for public release or provided as supplementary information upon publication. A list of possible software for analysing NGS data has been provided by Yu and colleagues (Yu et al, 2021) and can be expanded with the addition of RaptRanker (Ishida et al, 2020).

Given the number of sequences that will be generated at the end of aptamer selection, it is also important to note how sequences for further validation were chosen. Were the top ten sequences chosen on percentage reads within all the sequences or based on enrichment across rounds? Were they based off different predicted secondary structure motifs or 3D structure prediction? Were sequences discounted due to similarity to sequences from previous enrichment cycles? Any predicted structures for selected aptamers should be presented in the results or supplementary data and the choice of software and virtual folding conditions listed in the methods section.

For sequences that are selected for detailed studies, it is very useful to present related sequences from the same affinity-enriched pool (that is, sequences within a sequence family). Alignment of such sequences, including with appropriate gaps, often leads to insights about conserved as well as variable positions within an aligned set, which can be used for covariation analyses (to look for recurring base pairing to support secondary structure predictions, for example), truncation experiments, and identification of positions likely to be critical for target binding. Analyses of such sequence families are now considerably enhanced with the advent of NGS.

VALIDATION OF APTAMER SEQUENCES

Following identification of putative aptamer sequences, a number of potential candidates are then chosen for validation of binding, both for specificity and affinity. First, oligonucleotides synthesised with these sequences can be verified using mass spectrometry for completeness. This service is usually available from the oligonucleotide synthesis provider. Authors should also detail whether the aptamers were purified prior to characterisation and by what method, desalted, High-performance liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE), *etc*. This verification is particularly crucial in the case of chemically-modified aptamers. Next, assays and buffer conditions should be listed if they are different to the selection conditions. Examples of considerations for select types of targets are listed below. Regardless, both quantitative assays, and sometimes also qualitative assays should be included. Different characterisation methods can give different Kd values (or show binding *vs* no binding) for a given sequence, underscoring the importance of assay method. Importantly, scrambled and/or point mutation controls should be used in all assays to ensure binding is caused by the specific interaction of an aptamer. Scrambled controls should also be included if/when the aptamer sequence is truncated. Ideal control sequences must be of the same chemical composition and the same length to the sequences being tested. If modified bases have been introduced into the sequence, these should also be incorporated into the control sequences.

Regardless of the assay, all conditions must be included, such as binding buffer constituents, conditions for heating/cooling step for the proper folding of aptamer structure in buffer, concentration of aptamer, time of incubation, temperature, and washing steps. Both qualitative and quantitative experiments should be repeated multiple times to ensure reproducibility and the number of technical and biological replicates should be reported. Experiments should at the minimum be reproducible within the laboratory that reports the initial results, and if possible, repeated by a separate experimenter to confirm inter-operator reproducibility. Anecdotally, an experiment occasionally works when performed by one researcher, but fails in the hands of another member of the laboratory. Ideally, these experiments should be blinded prior to and during analysis to prevent results fitting preconceived expectations. This is especially important when images, such as those in histochemical applications, are presented that may not be representative of the entire population. For example, when taking images of cells, consecutive fields should be studied and images should be taken of each field to ensure a lack of bias. Finally, experimental protocols should detail the data analysis steps utilised to calculate binding affinities and selectivities (Table 3).

Aptamers that bind to small molecules

Small molecules often require different selection conditions compared to those typically used with protein targets. Based on structural analyses, aptamers generally encage small molecules through binding sites that contain contacts with multiple functional groups (Hermann and Patel 2000). With proteins, in contrast, high affinity binding and specificity is achieved through exquisite shape complementarity between aptamer and protein surfaces (Gelinas et al, 2016). The use of different immobilisation strategies may limit the aptamer binding to the free target or generate aptamers that only bind the small molecule attached to the matrix. Therefore, it is imperative that characterisation includes assays that replicate the selection process as well as those that replicate the future applications of the aptamers (Yu et al, 2021). Specifically, aptamers that were selected to immobilised small molecules should also be tested in solution using assays that do not require immobilisation such as isothermal titration calorimetry (ITC) (Chatterjee et al, 2020), microscale thermophoresis (MST), fluorescence anisotropy or by a molecular beacon fluorescence resonance energy transfer (FRET) assay (Endoh et al, 2009, Entzian and Schubert, 2016, Li and Zhao, 2019).

Given the challenges of immobilised small molecules, methods that do not require chemical changes in the small molecule targets are now strongly preferred, particularly since some small molecules have limited functional groups for immobilisation or immobilisation is difficult (Tian et al, 2019; Chatterjee et al, 2020; Lyu et al, 2021). If small molecules are immobilised by a chemical reaction, conditions must be reported in detail. This is to ensure that appropriate functional groups have been used for immobilisation.

Noteworthy examples for immobilisation free SELEX include Graphene Oxide (GO-) SELEX and Capture-SELEX (Park et al, 2012; Stoltenburg et al, 2012; Boussebayle et al, 2019). Both are based on structural change upon ligand binding with subsequent elution of binding sequences. GO-SELEX utilises the unspecific binding of DNA/RNA molecules, while Capture-SELEX uses a capture-oligonucleotide with a matching docking-sequence within the randomised pool sequence. Consequently, Capture-SELEX requires a special pool design which should be reported in-depth. In both cases, relevant specifications include the method of pool binding to the substrate/oligonucleotide with time, temperature and used buffers. Binding to matrix or used beads

Table 3. Minimum requirements for post-SELEX assay protocols.

General assay requirements	**Aptamer sequence**
	Chemistry (modifications, fluorescent dyes at 5’ or 3’, *etc.*)
	Aptamer purification
	Buffer and pH
	Folding conditions of aptamer
	Additional constituents
	Storage conditions of all reagents
	Target details
	Quantitative characterisation of binding or activity
	Positive and negative controls
	Number of biological and/or technical replicates
	Temperature
	Incubation time
Cell based assays	Adherent or suspension cells?
	Proliferating or quiescent cells?
	If adherent, what was used to detach cells?
	If trypsin used, were cells given a period of recovery prior to assay?
	Was fixation used?

must be excluded with appropriate experiments. Since the presence of matrix or capture oligonucleotides may influence binding characteristics, binding to the ligand should be demonstrated with and without the respective matrices, beads or oligonucleotides.

Aptamers that bind to proteins

Although there have been many adaptations to the SELEX process and targets are becoming more complex, the majority of aptamer selections are still directed against proteins, whether in their native conformations (*e.g.*, when expressed on the cell surface, for protein receptor targets, see also next paragraph below) or expressed as recombinant protein. A key consideration then when using recombinant proteins as the target is to ensure that the sourced proteins closely resemble the physiological state. Due consideration should be given to the folded conformation of the proteins and to any post-translational modifications (Díaz-Fernández et al, 2018). This is especially important since aptamers are now recognised as affinity reagents that have intrinsic ability to recognise even subtle differences in conformational states of proteins, with much higher sensitivity that what is achievable with antibodies (Zichel et al, 2012; Jankowski et al, 2020).

It is important, as with small molecule aptamers, to characterise aptamers under conditions of pH, temperature, and ion composition, that are similar to the ones used during their selection and that resemble the physiological milieu that will be found in future biomedical applications, such as blood, serum, urine, saliva, *etc*. For unmodified proteins, aptamers may bind to both recombinant proteins as well as to protein isolated from biological samples. However, for those proteins that undergo post-translational modifications, it is necessary to confirm the aptamer binds to the desired proteoform in proposed applications. This may involve the transfection of null cells with a tagged cDNA to allow for pull down of the protein following lysis. The protein should be confirmed via additional analysis, such as western blot, prior to assays. While some of the assays proposed for small molecule characterisation can also be applied to proteins, notably Surface Plasmon Resonance and MST, there are other assays that only require the use of a plate reader, either standard or fluorescent, for an enzyme-linked apta-sorbent assay (ELASA, also known as ELONA and ELAA (Drolet et al, 1996; Stoltenburg et al, 2016; Moore et al, 2017; Vargas-Montes et al, 2019) or fluorescence readout. The latter can be accomplished purely using fluorescently labelled primers, quantification, and denaturing and folding of the PCR product and subsequent incubation with the immobilised protein. Specificity of aptamer-protein recognition is extremely important and should be validated against constituents of the biological matrix in which the aptamer is intended to be applied. For example, many aptamers will be used to identify proteins in serum, plasma or blood samples or they will be developed for therapeutic purposes. The ability of the aptamer to bind the major protein constituents of the appropriate matrix should be tested and specified. Also, to the extent that the assay protocol allows, the aptamers should be tested for affinity to the target protein in the presence of the appropriate biological matrix, and with due consideration given to a series of positive and negative protein controls.

Aptamers that bind to cells

If developing aptamers for future cell-based assays, the aptamer should be tested against several cell lines that are positive for the target to determine the binding affinity. The aptamers should also be tested against cell lines that are negative for the target to confirm specificity. Given the complexity of the target, it is desirable to harness different types of assays in order to assess the targeting efficiency of the aptamers both in terms of affinity and specificity (*i.e.*, quantitative polymerase chain reaction (qPCR), flow cytometry, streptavidin-biotin-based assays). Flow cytometry represents a powerful analytical technique to determine aptamer cell binding and validate their target specificity. Light microscopy can be used as a complementary tool to define intracellular fate and localisation of aptamers upon receptor-mediated endocytosis or in some cases macropinocytosis with image collection and analysis by individuals blinded to the identify of each sample (Shigdar et al, 2011). These results should also be confirmed using both cell lines that express the target of interest (positive controls) and cell lines that do not (negative controls), ideally through the use of artificial expression and/or knockdown of the protein. If this is not possible, cell lines with a range of expression, from high to low expression should be used. The combined use of flow cytometry and light microscopy can demonstrate specific aptamer binding to the cell surface and/or its internalisation. If receptor-mediated endocytosis is proposed as the route of entry into the cell, several assays should be performed that (i) demonstrate colocalisation (aptamer co-incubation with endocytic markers, such as Rab-4, -5, -7 or transferrin), (ii) prevent endocytosis (sodium azide or potassium depletion, or use of inhibitors of clathrin-mediated endocytosis, such as Pitstop2 or Dynasore), or (iii) remove cell surface proteins (upon enzymatic digestion with trypsin or proteinase k) or digestion of surface bound aptamers using a cocktail of RNases or DNases. Blinded experiments as mentioned above are especially important when images are presented that may not be representative of the entire population. For example, if taking images of cells, consecutive or randomly chosen fields should be studied and images should be taken of each field to ensure a lack of bias.

Another consideration is the potential for aptamers to be taken up by dead cells non-specifically. This phenomenon has been reported in the literature, with methods proposed for the removal of dead cells (Mayer et al, 2010) to more accurately reflect the affinity of aptamers for cell expressed targets. Failure to account for non-specific uptake of aptamers by dead cells during aptamer affinity studies can result in misleading research findings (Flanagan et al, 2021).

Aptamers proposed for *in vitro* diagnostic applications

If an aptamer is developed for a disease specific and clinically relevant biomarker then, to demonstrate the real clinical utility of an aptamer candidate, it should be first evaluated in simulated samples (a pool of relevant biological fluid from healthy volunteers spiked with known concentration of biomarker). Following the initial "SELEX" publication, several other assays must be performed to bring this aptamer into the clinic. However, the authors note that these experiments would typically be in follow-up reports. First, the performance of the aptamer should be assessed in real clinical samples with a sufficient number of cases and controls to support statistical significance (Dhiman et al, 2018; Lavania et al, 2018; Kumari et al, 2019). The diagnostic sensitivity and specificity of the aptamer-based assay should be determined using a Receiver Operating Characteristic (ROC) curve to benchmark the performance of the aptamer with the existing gold standard test (Lavania et al, 2018; Taneja et al, 2020). Furthermore, a direct comparison of the performance of the aptamer with that of available poly/monoclonal (preferably monoclonal) antibodies in same set of clinical specimens is desirable for assessment of utility and to potentially highlight the superiority of aptamers over antibodies in the identified diagnostic assay.

Aptamers for *in vivo* applications

This field includes a wide range of applications including *in vivo* imaging and therapeutics. An aptamer used for an *in vivo* application needs to be carefully designed and for this purpose SELEX methodology as well as the post-SELEX modifications are critically important. For all *in vivo* applications, starting libraries that have some degree of intrinsic nuclease resistance have obvious advantages since they minimise the amount of post-SELEX optimisation to achieve desired metabolic stability. Selections done at physiological temperature, ionic strength and in buffers that contain divalent metal ions (calcium and magnesium) are strongly preferred for *in vivo* applications. Prior to initiation of *in vivo* studies, the *in vitro* affinity and specificity should be established by at least two different methodologies to ensure responsible use of animals. *In vivo* evaluation should also include: (i) the animal number in terms of 3 R's (Replacement, Reduction, Refinement) which restrict the procedures and cost (MacArthur, 2018); (ii) characterisation of stability in a biological fluid like serum; and (iii) dosing justification in the context of anticipated *in vivo* activity.

All points discussed here require optimised experimental design. Although there are additional biological barriers in an *in vivo* experiment, we expect the affinity and specificity of the aptamer to remain unchanged in the biological environment, especially if *in vitro* evaluation was performed with consideration of the physiological parameters. Serum stability assayed by size exclusion-high-performance liquid chromatography (SE-HPLC), gel electrophoresis or another suitable method is highly desirable. However, there are several parameters that define *in vivo* binding of the aptamers. If chemical modifications like dyes are included in the construct of the aptamer for *in vivo* applications, changes in overall lipophilicity should be considered because of the possibility of unspecific uptake in tissues. Also, usually aptamer size and composition may allow tissue penetration in hours. Thus, depending on the study, the optimal time for assessment of binding to the target *in vivo* may be after sufficient time is allowed for tissue penetration, which could be after around two or more hours (Bouvier-Müller and Ducongé, 2018). Various constructs have been used over the years to modify the residence time of aptamers *in vivo* such as polyethylene glycol, lipids such as cholesterol and nanoparticles. Most of these conjugates increase the effective size of the aptamers, which are much smaller than antibodies, by minimising their kidney-mediated clearance. Such constructs are very useful for a wide range of *in vivo* applications, however, it is essential to establish that such modification of the aptamer do not affect the binding affinity of the aptamer (Hilderbrand and Weissleder, 2010). Between 3 and 5 animals are typically recommended for each condition tested. Careful consideration should be given to the timing of the assessment of the effect of the aptamer in the context of the dosing schedule and the expected (or established) pharmacokinetic properties of the aptamer construct used for *in vivo* experiments. The choices for experimental design depend highly on the evaluation methodology. We recommend starting with few variables to evaluate the *in vivo* binding and target attainment. Sensitivity of the binding detection methodology must be considered (Sicco et al, 2020). Finally, a sequence-scrambled control reagents that have identical composition to active aptamer test agents should be included with all animal experiments (Haubner and Decristoforo, 2011). If the experiment includes aptamer modifications, the scrambled sequence needs to include the same modification. When feasible, a group of control of animals lacking the expression of the target should also be tested.

CONCLUSIONS

These guidelines are not exhaustive and cannot anticipate every situation experienced by authors, reviewers, and editors. However, it is our hope that this article will start a conversation about the minimum reporting guidelines required for publishing de novo aptamers to ensure that we stay ahead of the reproducibility crisis that has been faced by several fields. Although this is not a suggestion to reviewers to request additional experiments, the minimum requirements should be adhered to while balancing available resources, and in some cases, intellectual property policies of academic institutions and companies. We hope that both reviewers and authors will use the checklist in Table

Box 1: Checklist for publications

☐ All details are present for aptamer selection as per table 1
☐ All details are present for aptamer selection cycles as per table 2
☐ All details pertaining to aptamer sequence identification are present and top sequences are presented in the supplementary information
☐ All details pertaining to any structure prediction performed are present in the methods and structures are present in figure format
☐ All details pertaining to validation of aptamers are present in the methods, including any changes to buffers or conditions
☐ Aptamers were validated against positive and negative targets (including matrix-specific targets) to confirm specificity
☐ Appropriate controls for all experiments have been included and results presented

3 and Box 1, as well as the suggested tables, when preparing and reviewing articles in the future. While those of us who work specifically with aptamers understand the quirks that can affect experimental results, it is essential that we move forward with consistency to ensure that the wider community is able to follow our protocols and successfully use aptamers in their research and development projects.

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