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TECHNICAL REPORT

A guide to using nucleic acid aptamers in cell based assays

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ABSTRACT

Aptamers are single stranded nucleic acid molecules that fold into complex three-dimensional conformations. They have been used for a number of years as replacements for monoclonal antibodies by a core group of researchers. However, now that more and more companies are offering aptamers commercially for research and development applications, there is a need for a 'how to' guide for researchers who are interested in using aptamers for their own applications. Within this article, we detail the critical concerns for working with aptamers, from what to dilute them in, concentrations and times to use for incubation steps, as well as a suggested protocol. We hope that this guide will prove useful for researchers who are trialling aptamers for cell based assays, and as a starting point for researchers investigating aptamers for other applications.

KEYWORDS: aptamers, how to, method, protocol, technique

INTRODUCTION

Nucleic acid aptamers are DNA or RNA sequences that exhibit high affinity and specificity towards multiple types of targets, including proteins, nucleotides, small molecules and cells (Ellington and Szostak, 1990; Robertson and Joyce, 1990; Tuerk and Gold, 1990). First described almost 30 years ago, these so called chemical antibodies are developed in vitro using systematic evolution of ligands by exponential enrichment (SELEX) technique (Ellington and Szostak, 1990). Aptamers form complex three-dimensional shapes that bind to target molecules in a similar manner as protein antibodies while offering additional advantages including small size, quick and inexpensive synthesis, versatile chemical modification for improved stability and targeting capacity, and low/no immunogenicity or toxicity (Zhou and Rossi, 2017). As such, aptamers can effectively substitute monoclonal antibodies in a myriad of applications, including immunophenotyping (Nozari and Berezovski, 2017), immunohistochemistry (Shigdar et al, 2013b), targeted drug delivery (Zhou and Rossi, 2017; Henri et al, 2018), and biosensing (Kaur et al, 2018). Due to their promising potential, aptamers garnered considerable interest initially, though as with other nucleic acid compounds (Baird, 2010; Krieg, 2011), interest waned (Zhou and Rossi, 2017). However, there is a recent a resurgence with many researchers interested in using aptamers for both therapeutic and diagnostic application. (Ilgu and Nilsen-Hamilton, 2016; Romero-López and Berzal-Herranz, 2017; Kaur et al, 2018). Moreover, with a number of companies offering aptamers commercially (Table 1) either as custom aptamers, licensed aptamers, or synthesis of aptamers according to specific sequences, it is now easier for researchers to consider using aptamers in their assays. As with many emerging technologies, there are a number of nuances to using aptamers, yet a detailed 'how to' guide for non-experts in aptamer-based assays is missing. What follows is a discussion of these nuances, as well as a suggested protocol.

Table 1. Companies offering aptamers commercially

Company	Role	Country	Website
AMS Biotechnologies	Synthesise aptamer sequences	UK	http://www.amsbio.com
Aptamer Sciences Inc	Generation of novel aptamers	South Korea	http://www.aptamersciences.com/mains.html
BasePair Biotechnologies Inc	Generation of novel aptamers	USA	https://www.basepairbio.com
IBA GmbH	Synthesise aptamer sequences	Germany	https://www.iba-lifesciences.com/home.html
Integrated DNA Technologies	Synthesise aptamer sequences	USA	https://sg.idtdna.com/pages
Tocris Bioscience	Synthesise aptamer sequences	USA	https://www.tocris.com
TriLink Biotechnologies	Synthesise aptamer sequences	USA	https://www.trilinkbiotech.com

CRITICAL PARAMETERS AND TROUBLESHOOTING

Aptamer reconstitution and dilution

One of the most important aspects of working with aptamers is the choice of initial reconstitution or dilution buffer. Depending on the manufacturer, the aptamers arrive either reconstituted or lyophilised. If lyophilised, the standard reconstitution buffer is either Tris-EDTA (TE) buffer (10mM Tris, 0.1mM EDTA, pH 7.5-8.0) or non-DEPC treated nuclease free water. Aptamers may be stored frozen at -20 °C or at 4°C. A study by Integrated DNA Technologies (IDT) (Integrated DNA Technologies, 2014) demonstrated that while the choice of solution for storage at -20 °C has limited effect on the stability of the aptamers, the aptamer will be more stable if stored in TE buffer at 4°C. If the aptamer is amine-modified, avoidance of Tris containing buffers is recommended as the amines in the Tris buffer will compete with the amines on the aptamer in NHS ester reactions. Also, if the aptamer is thiol-modified a reducing step using TCEP (tris(2-carboxyethyl)phosphine) followed by further column purification would be required (de Castro et al, 2017).

With regard to diluting the aptamer to a working concentration, this is very much dependent on the aptamer selection conditions. Salts have a major effect on how aptamers, both RNA and DNA, fold into their complex three-dimensional shapes ready for binding to their target. There has been a range of selection buffers used by researchers and commercial companies, including Tris buffered saline (TBS), phosphate buffered saline (PBS), Dulbecco's PBS (DPBS), cell culture media, or complex solutions such as blood or serum. McKeague et al (2015) discussed the selection parameters used across approximately 500 aptamer selections to examine the effect that buffers or metal cations may have on binding sensitivity. Metal cations have been shown to significantly affect the secondary structure, stability, and affinity of aptamers (McKeague et al, 2015). From our own personal experience and others (Bai and Zhao, 2017), even small changes in the concentration of magnesium chloride can affect the affinity of the aptamers.

In summary, there is no standard buffer to dilute aptamers in for preparation and binding and thus care must be taken to use the exact same composition of salts in the preparation and binding buffers as those used for selection to ensure the correct folding and stability of aptamers. If purchasing from a commercial source, a data sheet should be provided with detailed information regarding the relevant solution to prepare the working concentration. Further to this point, if purchasing the aptamer from a company that specialises in nucleic acid synthesis (such as IDT or Sigma-Aldrich), HPLC purification should be chosen and the quality control analysis should demonstrate at least 90% purity. Lower purity will result in incorrect concentrations being used in assays and lead to false negatives. However, if a sequence is chosen from a published article or other source, it is imperative to begin initial validation studies using the same solutions that the research group chose.

Aptamer concentration

There is typically a wide range of suggested dilutions in the data sheet that comes with commercial antibodies. Aptamers have a much shorter range of working concentrations for cell based assays than antibodies. For example, antibodies might be used at a 1:500-1:1000 dilution or 5-10µg/ml for a particular assay, whereas aptamers diluted 1:50-1:200, or ~ 34-138ng/ml would give similar results. Similar to working with antibodies, several concentrations will need to be tested and validated which can be suggested from the paper in which they have been published, or from the company the aptamer was purchased from. In our experience, the optimal working concentration is 1.5-2 times the binding affinity of the aptamer measured as equilibrium dissociation constant or K_D, denoting the concentration at which 50% of the binding sites are saturated (Lambert, 2004). For example, if an aptamer has a binding affinity of 80nM with a specific cell line that expresses the target, a working concentration to trial would be 75nM, 100nM, 150nM, and 200nM. Validation is required as the aptamer may recognise a specific epitope or isoform of the target protein, the presentation of which is dependent on cell line expression. Aptamers may also have been selected to a purified or soluble protein, which may be conformationally different to the protein structure expressed by cells and thus, must be validated in appropriate in cell lines (Weller, 2018). Depending on the aptamer, its selection parameters, and K_D, it is likely that concentrations higher than 500nM will result in non-specific binding and should generally be avoided, though as is the case with low affinity antibodies, this will need to be determined for each individual aptamer. For example, we investigated one aptamer targeting a cell surface protein associated with cancer (Figure 1). Using our standard conditions as detailed in the protocol, we determined that

the binding of our aptamer up to 200-400nM was specific to the target cell line but then became 'sticky' and binding became non-specific above this concentration. Note that as aptamers can be internalised readily into live cells, we perform our incubation steps at 4°C and may include 0.1% (w/v) sodium azide to limit receptor-mediated endocytosis if staining for a cell surface receptor.

Blocking non-specific binding sites

Cells have non-specific binding sites that require blocking prior to incubation with a specific targeting agent. In the case of antibodies, this typically involves serum or bovine serum albumin (BSA). For aptamers, this step also involves incubation with tRNA or salmon sperm (DNA) to block nucleic acid binding sites. Our protocol uses tRNA at a concentration of 0.1mg/ml. Of note, nucleic acids are negatively charged and may attach non-specifically to positively charged proteins. We, therefore, use BSA in our blocking and binding buffers as it is negatively charged at neutral pH (Phan et al, 2015) and will potentially block positively charged sites. In the case of cancer cells, a recent study has shown that cancer cells typically have a negative charge whereas normal healthy cells have a neutral or positive charge (Chen et al, 2016; Le et al, 2019) so the use of BSA is highly recommended for use in assays detecting biomarkers in non-cancer cells, or when testing specificity. As metal cations can help to mask the negative charge of the DNA backbone, higher concentrations of $MgCl_2$ may be found in assays detecting biomarkers on the surface of cancer cells (McKeague et al, 2015). This could also explain why changes in magnesium chloride concentration have an effect on binding affinity.

Aptamer folding

Compared to the use of the antibodies which only require a simple dilution prior to application, aptamers require an additional step to fold them into their three-dimensional conformational shape. This typically involves a heating step to denature the single stranded species to allow for the aptamers folding into a complex shape specific for binding to the target. A typical protocol for DNA aptamers is to heat to 85°C for 5min, followed by a further incubation at room temperature and sometimes followed by an incubation at 37°C (Macdonald et al, 2016). The protocol may also suggest incubation on ice following the initial denaturation step. It should be pointed out that the choice of tube and heat block or PCR machine can influence the folding of the aptamer. Heat conductivity through thick walled plastic tubes, such as 1.5ml Eppendorf tubes is not as efficient as thin walled PCR tubes. If your only choice is

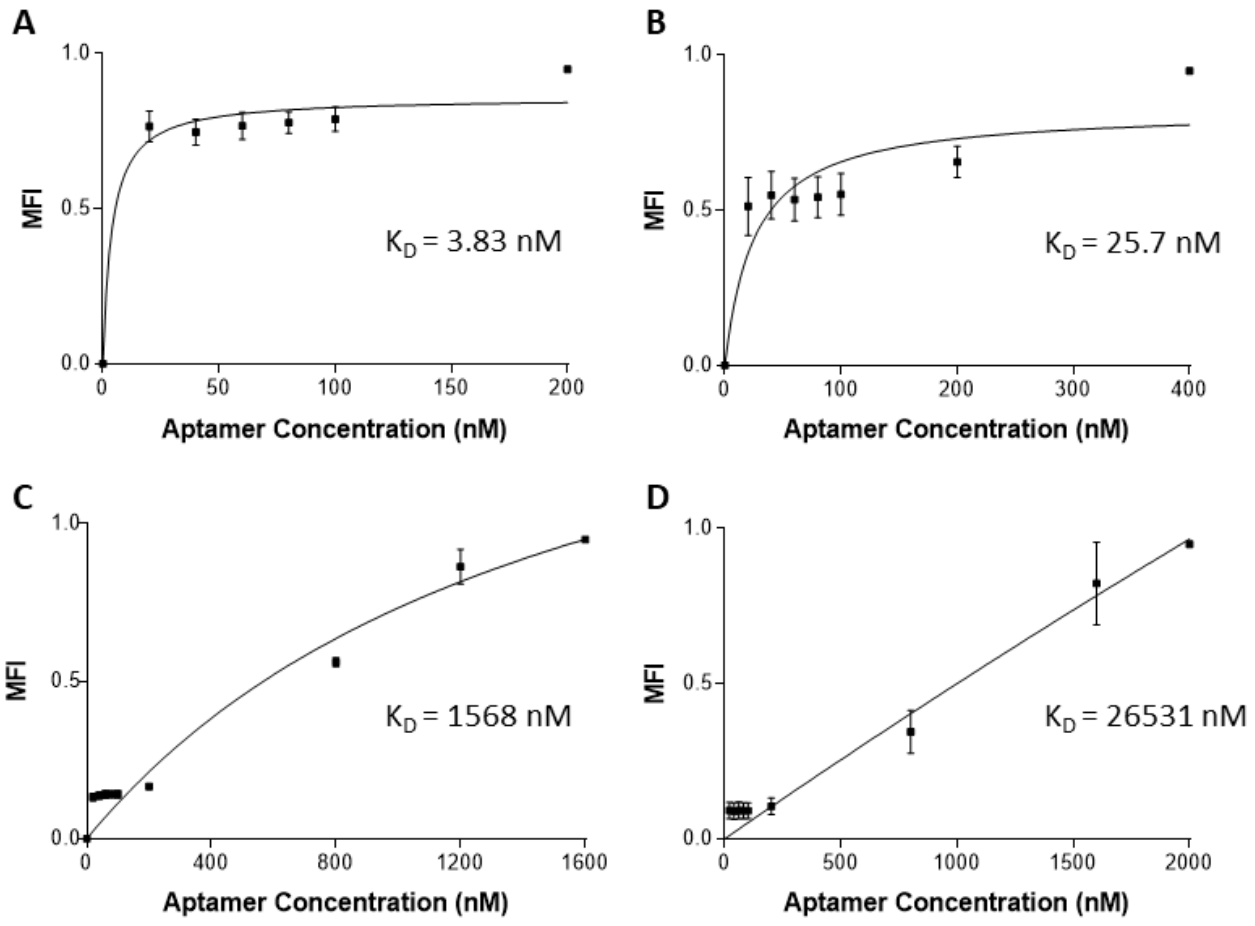

Figure 1. Aptamers demonstrate non-specific binding at high concentrations. Binding conditions are presented in the protocol. K_D was assessed at varying concentrations at 4°C for 30min: A: 0-200 nM; B: 0-400 nM; C: 0-1600 nM; D: 0-2000 nM. MFI: Median fluorescence intensity

a 1.5ml tube and a heat block, then longer than 5 minutes is suggested at 85°C to ensure denaturation of the single stranded species. Additional time is also required for the other folding steps. Our protocol, detailed below, suggests 5 minutes at 85°C, followed by 10min at room temperature, and 15min at 37°C. A pre-cycle is also suggested to ensure PCR machine is adequately heating to correct temperatures as older PCR machines may be inefficient when first turned on. We prefer to anneal our aptamers immediately prior to the assay and keep the aptamers at their final 'folding temperature' until addition to cells.

Aptamer incubation times and temperatures

One of the beneficial properties of aptamers is that they tend to bind to their target quite quickly, which is usually due to the selection parameters. Most aptamer selections start with a 1hr incubation which then is reduced to 30min or less during the iterative cycles (Rahimizadeh et al, 2017). Therefore, validation should also include different incubation times, depending on the assay. A typical suggestion for most aptamers would be 20, 30, and 60mins, though guidance should be taken from the publication or commercial company. A longer than suggested incubation time may result in no staining due to fast off-rates so more is not necessarily better. It is recommended to assess cell binding and uptake of the aptamers by fluorescent or confocal microscopy using a positive and a negative control. This can allow a range of concentrations and times to be tested. Incubation temperatures will however depend on the assay. For example, aptamers can be readily internalised via receptor mediated endocytosis so if the application is to assess the expression of a cell surface marker on a range of cell lines or patient samples via flow cytometry, then temperature is an important consideration. For these types of applications, incubation should be performed on ice or at 4°C as is the case with antibody protocols for cell surface receptors. If testing to see if an aptamer is internalised under physiological conditions, then 37°C would be preferred. It is worth noting though, that the incubation temperature can be aptamer-dependent and that aptamers selected for at a specific temperature may not show binding at other temperatures (Mallikaratchy et al, 2011; Wang et al, 2017). Again, it is important to recapitulate the selection conditions when first validating an aptamer.

Functionalisation of aptamers

One emerging application for aptamers is as targeting moiety to deliver drugs or nanoparticles to specific cells. This requires functionalisation of the aptamers with specific chemistries to allow for stable conjugation with the cargo. While modifications to any of the nucleotides within the aptamer can affect the specificity or sensitivity (Shigdar et al, 2013a), attachment of molecules, such as an amine or thiol group, to the end of the aptamer may not affect their properties, thus all modifications should be assessed on a case-by-case basis. If a drug such as doxorubicin, which intercalates into the double stranded stem of the aptamer, is being used then this may affect the tertiary structure of the aptamer and potentially affect its specificity and sensitivity. Additionally, increases in size of the aptamer-cargo complex may affect the rate of cellular internalisation and therefore, incubation times will need to be reassessed. Validation of binding specificity and sensitivity should ideally be performed with the non-functionalised aptamer, the aptamer with the modification, and the aptamer in its final form with the drug or nanoparticle attached.

Quality control testing

Some aptamer preparation protocols, such as the reducing reaction for thiolated aptamers, require a purification step. Therefore, analysis of the concentration and stability is required. As aptamers are DNA or RNA, this can be completed in a similar manner to quantifying a PCR product, using UV spectroscopy (Barbas et al, 2007), NanoDrop (Desjardins and Conklin, 2010) or newer technologies such as Qubit fluorometric determination (Nakayama et al, 2016). Stability can be assessed using polyacrylamide gel electrophoresis (Kratschmer and Levy, 2017). Aptamers are typically more stable than antibodies so transport conditions and storage will have less impact on their usability but if an aptamer has been stored for long periods of time, it is wise to confirm that the sequence is still intact through the use of gel electrophoresis. A single sharp band should be visible (Li et al, 2017). Additionally, as aptamers are single stranded nucleic acids, a denaturation gel will need to run by including a denaturation agent in the buffer such as urea (Summer et al, 2009). Like most gel electrophoresis with nucleic acids, smearing and multiple banding may be an indication of degraded aptamer over time, likely from exposure to environmental nucleases.

Protocol for aptamer binding assay using fluorescently labelled aptamers

This protocol presents a basic step by step guide to a cell based assay for aptamers as a starting guide to help optimise protocols. Additional detailed information and further reading can be found in Sefah et al Nature Protocols paper (Sefah et al, 2010). Care must be taken to use nuclease-free reagents and solutions in all steps of the assay for nucleic acid aptamers. We have used this assay for both DNA and RNA aptamers of lengths ranging from 19 nucleotides to 90 nucleotides (Shigdar et al, 2011; Shigdar et al, 2013c; Macdonald et al, 2017).

*Note 1: as nucleic acid aptamers are similar to PCR primers, they can be tagged with any fluorophore. We have found no differences with 5' or 3' labelling though preference should be given to the end without a guanine residue due to fluorescent quenching (Mao et al, 2018).

1. Using ~ 200,000 cells per tube, cells are incubated with blocking buffer for 30min at 4°C. The blocking buffer consists of Dulbecco's phosphate buffered saline (DPBS) with 5mM $MgCl_2$ (See note 2), 10% (v/v) fetal calf serum (FCS), 0.1mg tRNA, and 1mg BSA per ml solution.

*Note 2: the DPBS and concentration of $MgCl_2$ is specific to the aptamer developed by the authors and will be specific for each aptamer.

2. Aptamers are prepared at a range of concentrations (20-400nM and folded using a PCR machine (85°C for 5min, 22°C for 10min, 37°C for 15min (see note 3)).

Typical volumes are 50-100µl of DPBS and $MgCl_2$ (See note 3).

*Note 3: the aptamer folding conditions are specific to each new aptamer developed, and the conditions that are outlined in the literature by the developing authors should be used for each new aptamer.

3. The blocking buffer is removed from the cells following a brief centrifugation step to pellet the cells and the cells are resuspended with aptamer. FCS (10%), BSA (1mg/ml) and tRNA (0.1mg/ml) is added to the solution (See note 4).

*Note 4: if BSA and tRNA are added to the aptamer prior to folding, the aptamer will not fold into its correct conformation and binding will not occur. Sodium azide can also be added to prevent internalisation.

4. The cells are incubated with aptamer for 30min at 4°C (See note 5)

*Note 5: the incubation time will vary with aptamer and the temperature may vary depending on the assay.

5. The aptamer solution is removed following a brief centrifugation and the cells are washed three times with DPBS containing 5mM $MgCl_2$ (See note 2).

6. Aptamer binding is assessed using flow cytometry, fluorescence spectroscopy, or fluorescence microscopy (See note 6).

*Note 6: Choice of equipment for analysis of aptamer binding is at the discretion of the user. Flow cytometry and fluorescence spectroscopy can provide information on aptamer binding but not location of the aptamer. Fluorescence microscopy can provide information on the location of the aptamer (on the cell membrane or internalised into the cell). A combination of methods is suggested to provide as much information regarding the aptamer as possible (Figure 2).

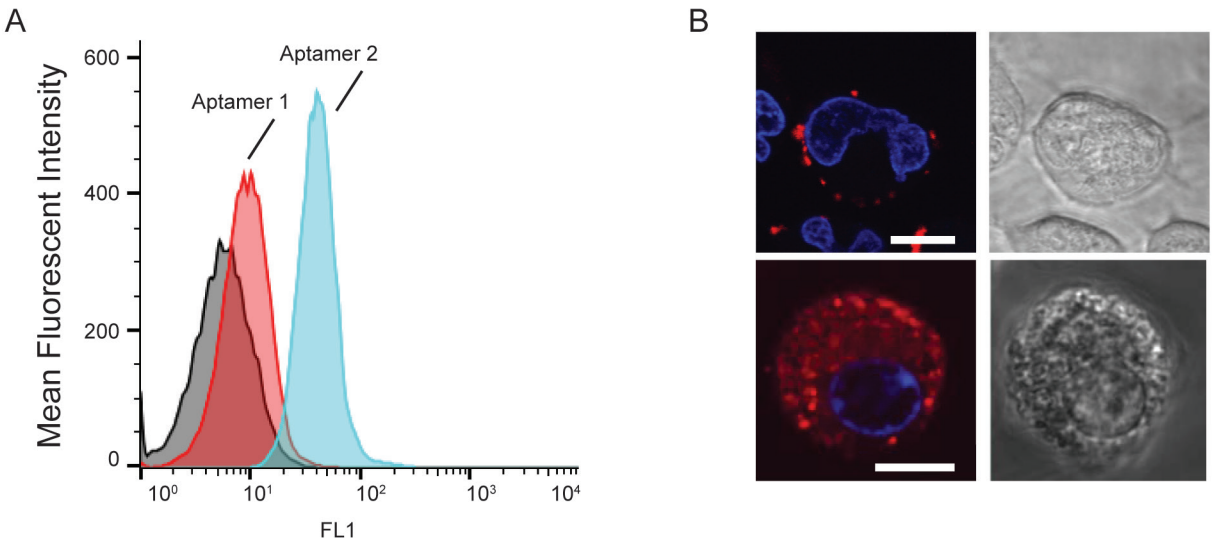

Figure 2. Assessment of aptamer binding can be analysed either quantitatively or qualitatively. A: Quantitative analysis of aptamers with different binding affinities. Aptamer 1 has a lower binding affinity to aptamer 2. Autofluorescence indicated by grey peak; B: qualitative analysis of aptamer binding at 4°C (top panel) and at 37°C (bottom panel) showing localisation to the cell membrane or internalisation throughout the cytoplasm, respectively. Scale bar = 10µm.

SUMMARY

It is now nearly thirty years since aptamers were first described, and while it has taken a while for them to find their niche, aptamers are starting to be utilised by researchers outside of the aptamer community. With a number of companies now offering aptamers for commercial sale, it is becoming easier for them to be used in many different applications. The authors hope that this guide will assist researchers to use these aptamers for better reproducibility in their assays in the future.

COMPETING INTERESTS

None declared.

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