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A Flow Cytometry-based Cell Surface Protein Binding Assay for Assessing Selectivity and Specificity of an Anticancer Aptamer

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Abstract

A key challenge in developing an anticancer aptamer is to efficiently determine the selectivity and specificity of the developed aptamer to the target protein. Due to its several advantages over monoclonal antibodies, aptamer development has gained enormous popularity among cancer researchers. Systematic evolution of ligands by exponential enrichment (SELEX) is the most common method of developing aptamers specific for proteins of interest. Following SELEX, a quick and efficient binding assay accelerates the process of identification, confirming the selectivity and specificity of the aptamer.

This paper explains a step-by-step flow cytometric-based binding assay of an aptamer specific for epithelial cellular adhesion molecule (EpCAM). The transmembrane glycoprotein EpCAM is overexpressed in most carcinomas and plays roles in cancer initiation, progression, and metastasis. Therefore, it is a valuable candidate for targeted drug delivery to tumors. To evaluate the selectivity and specificity of the aptamer to the membrane-bound EpCAM, EpCAM-positive and -negative cells are required. Additionally, a non-binding EpCAM aptamer with a similar length and 2-dimensional (2D) structure to the EpCAM-binding aptamer is required. The binding assay includes different buffers (blocking buffer, wash buffer, incubation buffer, and FACS buffer) and incubation steps.

The aptamer is incubated with the cell lines. Following the incubation and washing steps, the cells will be evaluated using a sensitive flow cytometry assay. Analysis of the results shows the binding of the EpCAM-specific aptamer to EpCAM-positive cells and not the EpCAM-negative cells. In EpCAM-positive cells, this is depicted as a band shift in the binding of the EpCAM aptamer to the right compared to the non-binding aptamer control. In EpCAM-negative cells, the corresponding bands of EpCAM-binding and -non-binding aptamers overlap. This demonstrates the selectivity and specificity of the

EpCAM aptamer. While this protocol is focused on the EpCAM aptamer, the protocol is applicable to other published aptamers.

Introduction

Cancer is still one of the leading causes of mortality worldwide[1]. Despite the significant improvement in cancer treatment in recent decades, anticancer drug development is still a highly debated topic. This is because chemotherapy, as the mainstay of cancer treatment, is accompanied by serious side effects that limit patient compliance with the treatment. Moreover, chemotherapy-induced cancer resistance to treatment has restricted its application as the sole choice of medical intervention. The application of monoclonal antibodies (mAbs) introduced an enhanced response to cancer treatments[2]. The rationale of using mAbs was to improve the efficacy of chemotherapeutics and minimize their adverse reactions. However, the administration of mAbs also became a challenge. This was not only because of the mAb-induced immunological reactions but also due to the animal-dependent and expensive production costs and difficult storage conditions[3]. Introduction of aptamers in the 1990s[4] raised new hopes in cancer treatment, as the application of aptamers could address the challenges associated with mAbs.

Aptamers are short nucleic acid sequences that are specifically produced for a certain target. Systematic evolution of ligands by exponential enrichment (SELEX) is a common method in aptamer production. In SELEX, the protein of interest is incubated with a library of random nucleotide sequences, and through a series of iterative cycles, the aptamer specific for that protein is purified. Aptamers have similar target selectivity and specificity to mAbs, and therefore drug development in this field shows promising future applications. Aptamers specific for cancer biomarkers could be applied as single drugs and cancer diagnostic tools[5,6,7]. Due to their nano-sized structure, these aptamers could also act as drug carriers to deliver cytotoxic agents specifically to the tumor[8]. This would increase the efficacy of targeted drug delivery and decrease chemotherapy-associated, off-target adverse reactions. Moreover, these nanomedicines have a high tissue penetration, which makes them a desirable candidate for deep-tumor drug delivery and treatment. Aptamers can also be designed to target the transporters expressed on the blood-brain barrier (BBB) to improve drug delivery to brain tumors[9]. A good example of such an aptamer are bifunctional aptamers, targeting the transferrin receptor (TfR)[10] to enhance drug delivery across the BBB, and delivering a cytotoxic drug payload to tumor cells[11].

Despite all the advantages of aptamers, drug development in this field has not yet yielded a marketed, successful anticancer drug. One reason for this could be the lack of standard and reproducible methods that could be followed globally by researchers in the field. In this paper, a step-by-step protocol of an aptamer binding to a native protein expressed on the cell surface is demonstrated. This protocol is a prerequisite step in the preclinical assessment of anticancer aptamers. The assay is performed to show the selectivity and specificity of the purified aptamer collected from SELEX or a published aptamer sequence for confirmation of selectivity and specificity. This flow cytometric-based assay is a rapid, reliable, sensitive assay that accurately shows the selectivity and specificity of the

aptamer, where the aptamer is being tested against proteins on the cell surface[12,13,14]. This method is demonstrated using the binding of an aptamer specific for EpCAM shown in this paper[15]. EpCAM, as a transmembrane glycoprotein, plays roles in tumor cell signaling, progression, migration, and metastasis[16,17]. To show the selectivity and specificity of this aptamer, EpCAM-positive and -negative cancer cells were used. The previously developed EpCAM specific aptamer, TEPP (5′-GC GCG GTAC CGC GC TA ACG GA GGTTGCG TCC GT-3′), and a negative control aptamer, TENN (5′-GC GCG TGCA CGC GC TA ACG GA TTCCTTT TCC GT-3), were used as EpCAM-binding and -non-binding aptamers, respectively[10]. The 3' end of both TEPP and TENN were labeled with a TYE665 fluorophore.

TEPP is a bifunctional aptamer that targets EpCAM from one end and TfR on the other. This has made TEPP a suitable candidate for drug delivery to $EpCAM^+$ brain tumors. Using its TfR-specific end, TEPP traverses the blood-brain barrier, and using the EpCAM-specific end, finds the tumor and delivers its cargo (e.g., cytotoxic drugs) to the tumor. TENN has a similar length and 2D structure as TEPP, but it does not have affinity for the EpCAM or TfR, and hence is a suitable negative control aptamer. Using TEPP and TENN, testing the binding of an aptamer to the target protein using flow cytometry is shown in this paper. This protocol applies to the development of cell-specific aptamers. It is also applicable to further complementary and confirmation analyses of the aptamer sequences available in the literature. The protocol can also be used by those new to the aptamer field who are looking at using a previously published aptamer for their research and development (R&D) purposes. In this paper, two aptamer sequences available in the literature are studied.

Protocol

NOTE: Prior to starting the experiment, wear personal protective equipment, including a lab coat, gloves, and goggles. See the **Table of Materials** for details about materials, reagents, equipment, and software used in this protocol.

1. Buffers required for the assay

1. Prepare the buffers required for this experiment-the SELEX buffer required for aptamer folding, Blocking Buffer (BB), Wash Buffer (WB), and Binding Buffer (BiB) (**Table 1**)-freshly on the day of the experiment and keep them on ice or at 4 °C.

   NOTE: Each aptamer requires a unique folding condition. This includes the SELEX buffer and folding temperature conditions. Care should be taken to fully replicate the methods from the original paper describing the aptamer[10]. In this experiment, all buffers are prepared in Dulbecco's phosphate-buffered saline (DPBS). The buffer volume required in each experiment depends on the number of cell lines, number of replicates, and number of aptamer concentrations that are tested.

	Ingredients		Volume required
	Item	Concentration	
SELEX buffer	$MgCl_2$	5 mM	50 µL per sample + 10% pipetting error
Blocking Buffer	$MgCl_2$	5 mM	500 µL per cell line

	BSA [a]	1 mg/mL	
	tRNA [b]	0.1 mg/mL	
	FBS [c]	10% (v/v)	
Wash Buffer	$MgCl_2$	5 mM	1 mL for the first wash + 100 µL per test sample + 10% pipetting error
Binding Buffer	$MgCl_2$	5 mM	50 µL per sample + 10% pipetting error
	BSA	2 mg/mL	
	tRNA	0.2 mg/mL	
	FBS	20% (v/v)	

Table 1: Buffers required for the binding assay. [a]Bovine Serum Albumin, [b]Transfer Ribonucleic Acid, [c]Fetal Bovine Serum.

2. Preparation of aptamers

NOTE: The aptamers used in the assay are tagged with a fluorescence reporter molecule, and therefore care should be taken to protect them from light.

1. Prior to the experiment, prepare a 100 µM stock (stock A) of test and control aptamers using pyrogen- and RNase-free ultrapure water (**Figure 1**).
   NOTE: For long-term preservation, stock A should be kept in a freezer at -20 °C.
2. Prepare stock B as the working concentration of aptamers by diluting stock A using SELEX buffer (**Table 1**). To follow this protocol, dilute stock A to a 1,000 nM stock to prepare stock B (**Figure 1**).
3. To make the aptamer ready for the formation of the 3-dimensional (3D) structure, in a 250 µL tube, dilute stock B with SELEX buffer to prepare the required volume and concentration of the aptamer for folding.
   NOTE: The folded aptamers will be exposed to an equal volume of cells. Therefore, the concentration of the aptamer that is set for folding should be 2x more concentrated than the desired final concentration. Use equation (**1**) to calculate the required volumes and concentrations. Remember to consider an extra 10% volume for the pipetting error.

$$Concentration_{stock\ A} \times Volume_{stock\ A} = Concentration_{stock\ B} \times Volume_{stock\ B} \quad (1)$$

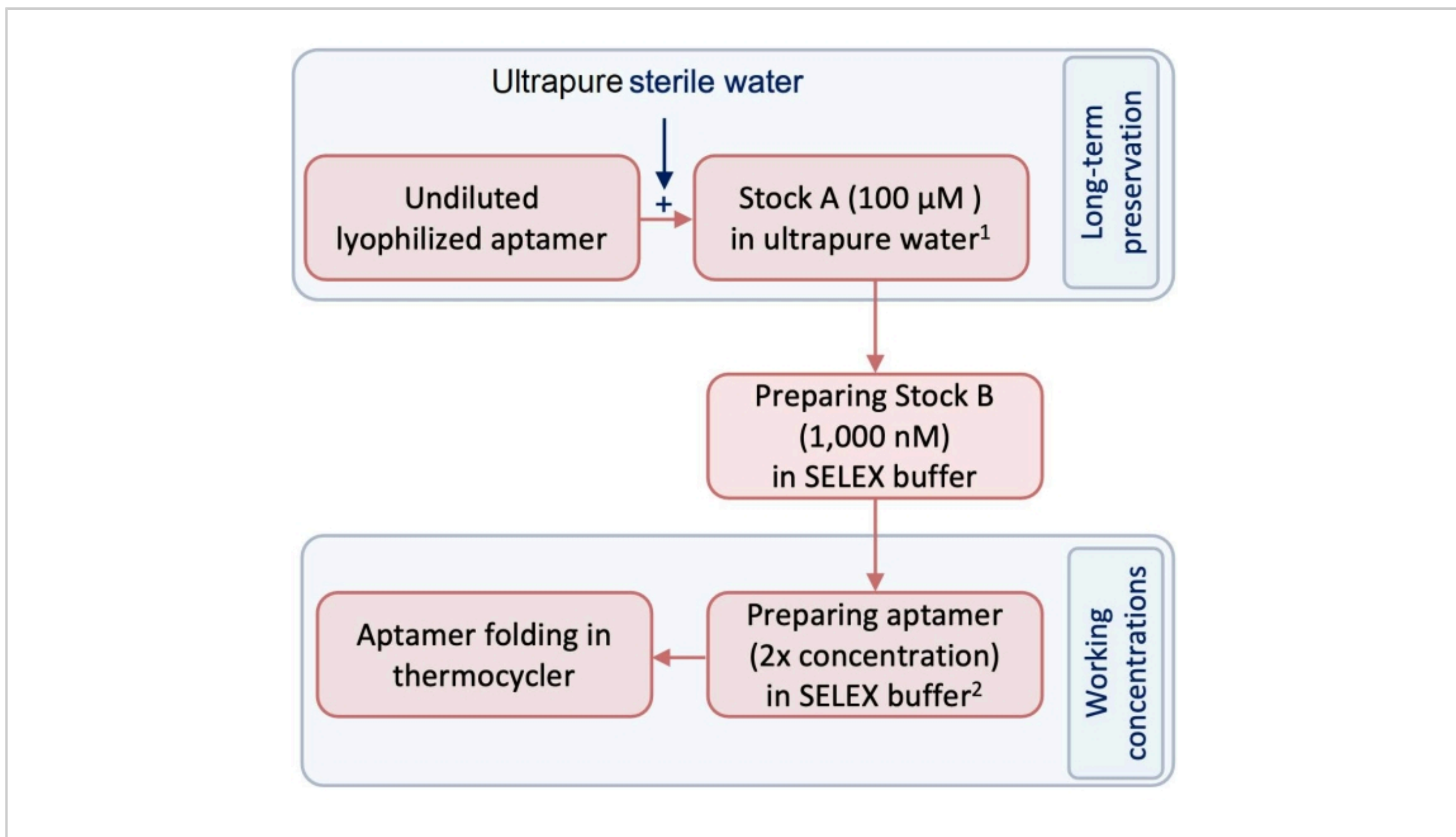

Figure 1: A diagram showing the steps in the preparation of aptamers. [1]Stock 1 is stored at -20 °C for long-term preservation. [2]Working concentrations are prepared in SELEX buffer and are not stored. Please click here to view a larger version of this figure.

3. Maintenance of cancer cells

NOTE: Prior to commencement of the study, make sure that the cells are at their early passage numbers, show their typical morphological features, and are mycoplasma free. To test the selectivity and specificity of the aptamer, cell lines that are high, moderate, and low/negative expressors of the protein of interest are ideally required.

1. Seed the cells in a T75 culture flask, using appropriate culture conditions. Grow them in a 5% CO_2 humidified incubator, at 37 °C.
   NOTE: In this study, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (complete medium) was used.
2. When the cells reach ~80% confluency, passage them to a new flask containing fresh complete medium.
   NOTE: Depending on the protein of interest and the cell line, 80% confluency could provide a suitable cell population for the binding assay. For the cell lines in this experiment, MDA-MB-231 and HEK 293T, 80%

confluency is suitable. At this stage, proceed to section 4, the binding assay. Always check the expression of the protein of interest, using mAbs specific for that protein.

4. Binding assay

NOTE: **Figure 2** summarizes the steps required in the binding assay in adherent cells.

1. In a class II biosafety cabinet, collect the cells of each flask in tubes as follows:
    1. Collect and discard the media in the flask, add 2 mL of PBS, spread it over the cells, and then, collect and discard the PBS. Repeat this step twice more to remove all traces of media that may inactivate trypsin. Add 1 mL of 0.25% of trypsin/EDTA to each flask and incubate for 5-10 min at 37 °C. Visualize the detachment of cells under a microscope.
    2. Add 1 mL of complete medium to the cells, and pipette the cells up and down to make a single-cell suspension. Pipette the cells into an appropriate tube and centrifuge at 200 × *g* for 5 min.
    NOTE: For non-adherent cells, collect the cells in a tube, centrifuge (200 × *g*, 5 min), and proceed to step 4.1.3.
    3. Discard the supernatant and resuspend the cells in 1 mL of fresh medium. Count the cells using trypan blue staining, by diluting a certain volume of cell suspension with trypan blue. Distribute ~15 µL of the mixture between a hemocytometer and a cover glass. Count the cells as previously described[18], using equation (**2**):

$$Total\ cells\ per\ mL = \frac{(Total\ cells\ counted \times 10{,}000\ cell/mL)}{Number\ of\ squares\ counted} \quad (2)$$

    NOTE: Use the minimum possible volume of cell suspension and take a note of the dilution factor. For example, mixing equal volumes of cell suspension and 0.04% trypan blue gives a dilution factor of 2. Ensure high viability (live cells/total cells × 100) of ~90% for most adherent cell lines before proceeding. Dead cells non-specifically take up aptamers and alter the results[19]. It is possible to use other cell counting techniques, such as using a cell counter.
    4. Collect the required number of cells, making sure to have 10 × 10^4 cells per test sample. Consider an extra 10% volume for pipetting error.
    NOTE: It is important to always keep the same cell count between experiments and replicates.
    5. Incubate the cells at 37 °C for 2 h to allow for the stabilization of the protein of interest on the cell membrane following enzymatic detachment.
    NOTE: This incubation period might differ according to the protein of interest.
2. During this 2 h incubation:
    1. Set the temperature of the centrifuge to 4 °C. Leave tRNA and stocks of aptamer at room temperature or on ice to thaw. To protect the fluorescence reporter molecule, protect the aptamer tubes from light.
    NOTE: The role of tRNA is to block the nucleic acid binding sites.
    2. Prepare the SELEX buffer, BB, WB, and BiB (see section 1), keeping them all on ice or at 4 °C. Set the thermocycler machine on an empty cycle. Place a 96-well black plate and flow cytometry tubes on ice.

NOTE: Setting the thermocycler on an empty cycle prepares the cooling and heating system and helps generate more reproducible results.

3. Following the 2 h incubation, centrifuge the cells at 500 × *g* for 5 min. Discard the supernatant and resuspend the cells in 500 µL of BB. Incubate the cells at 4 °C for 30 min with intermittent mixing.

4. During this 30 min incubation, perform aptamer folding as follows:

    1. Make up the 2x concentrations of aptamers (see section 2), and then mix and incubate the aptamers in the thermocycler machine, according to the required folding conditions. For this EpCAM aptamer, use the following folding conditions of 95 °C, 5 min, followed by 22 °C, 10 min, and 37 °C, 15 min.

    NOTE: Always include a negative control (i.e., SELEX buffer without aptamers).

5. Following the 30 min incubation, centrifuge the cells (500 × *g*, 5 min, 4 °C), remove the supernatant, add 1 mL of WB, and centrifuge the cells again (500 × *g*, 5 min, 4 °C). Remove the supernatant and resuspend the cells in a suitable volume of the BiB.

6. Pipette 50 µL of the resuspended cells into each well of an ice-cold, 96-well black plate. Keep the cells on ice to inhibit internalization of the protein of interest.

7. Pipette 50 µL of the aptamers onto a 50 µL volume of cells, mix, and incubate in darkness at 4 °C for 30 min. Centrifuge the plate at 500 × *g*, 5 min, 4 °C, and carefully remove the supernatant.

8. Carefully resuspend the pellet in WB and centrifuge at 500 × *g* for 5 min. Repeat the wash step (4.7) 2x and resuspend in 100 µL of WB for flow cytometric analysis.

NOTE: See **Figure 3** for a diagram of interactions between aptamers and cells.

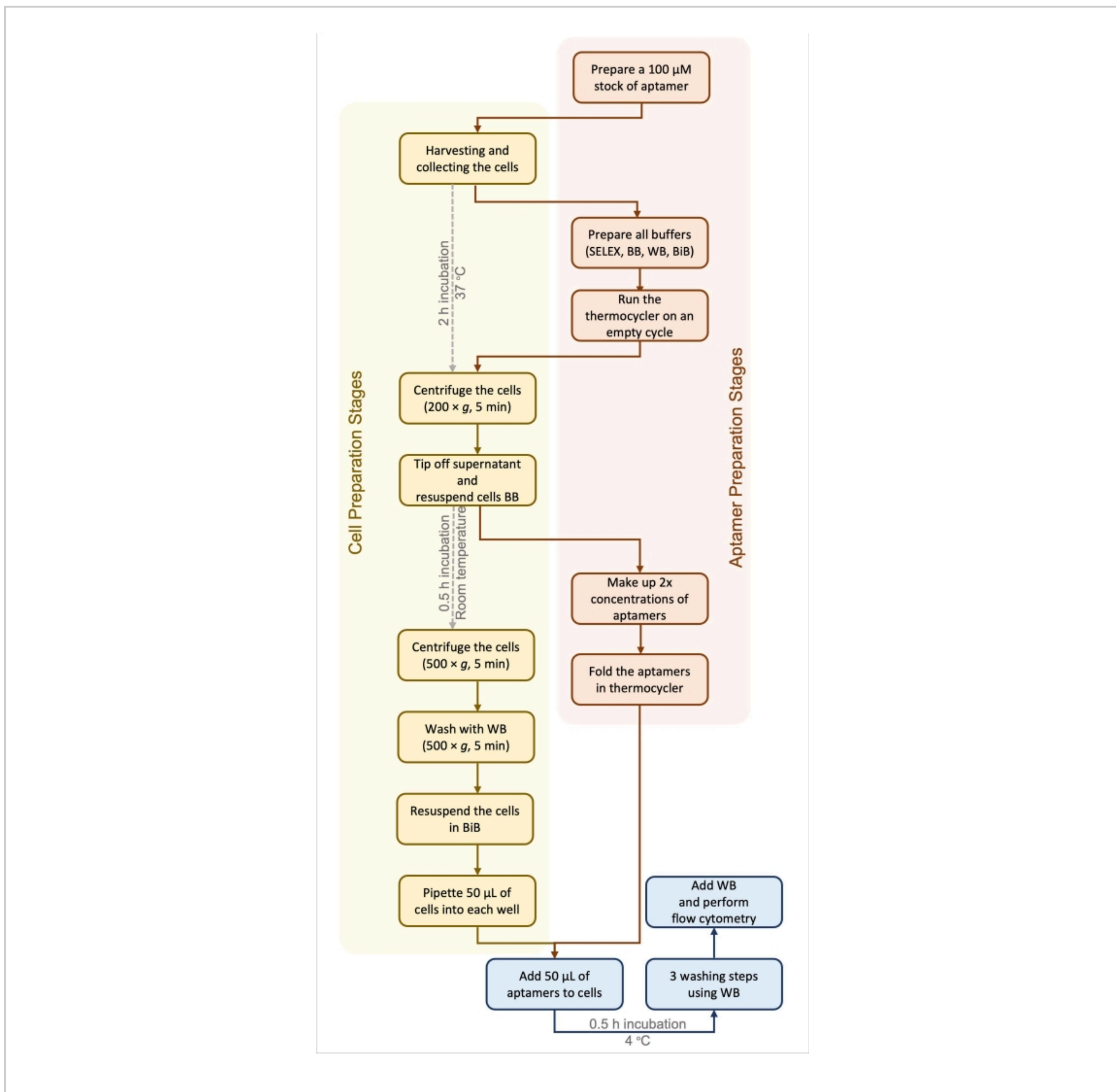

Figure 2: A diagram depicting the steps in performing an aptamer-protein-binding assay. Abbreviations: SELEX = Systematic Evolution of Ligands by EXponential Enrichment; BB = Blocking Buffer; WB = Wash Buffer; BiB = Binding Buffer.

Please click here to view a larger version of this figure.

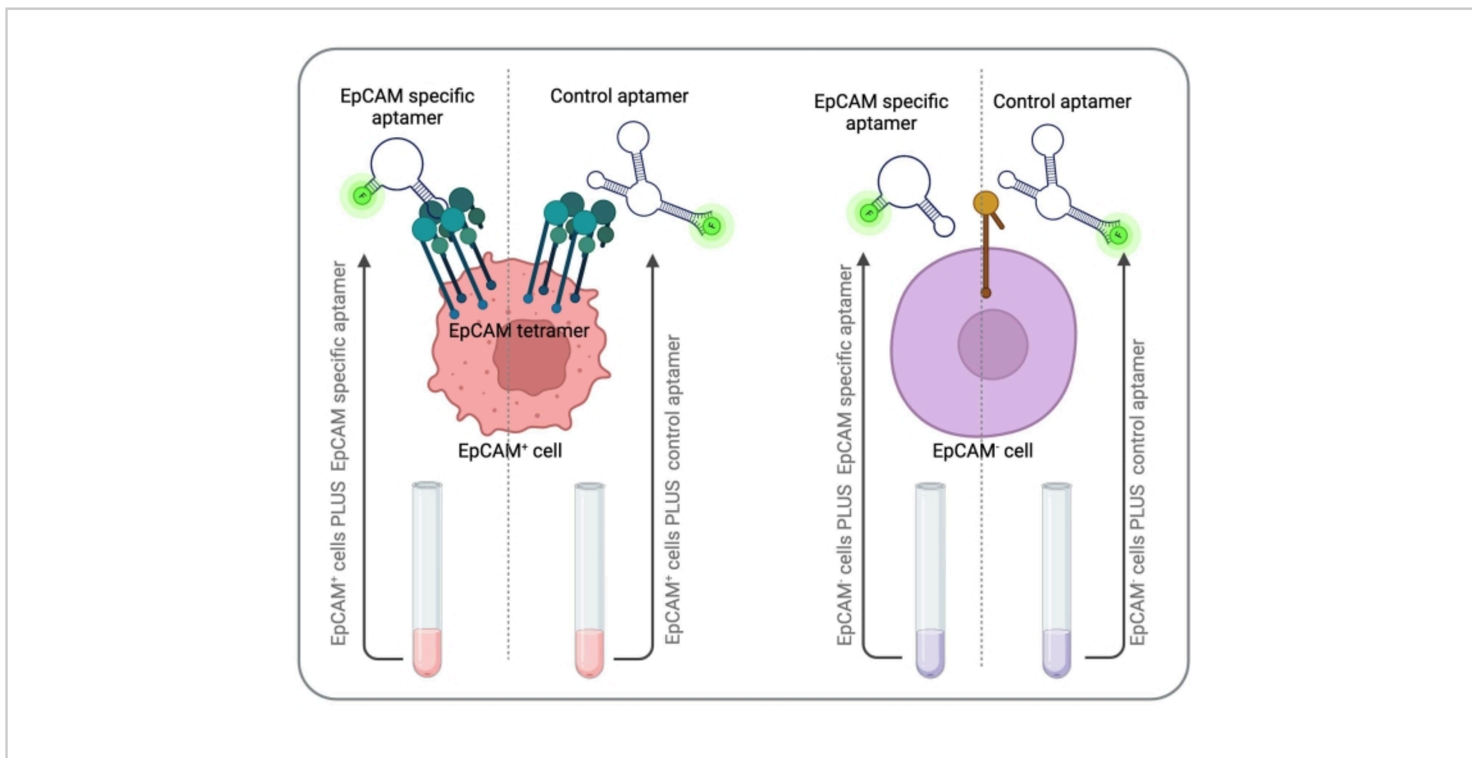

Figure 3: A diagram showing the different types of cells and aptamers required to perform the aptamer binding assay. Abbreviation: EpCAM = epithelial cellular adhesion molecule. This figure was created using Biorender.com. Please click here to view a larger version of this figure.

5. Flow cytometry and data analysis

NOTE: Before turning on the flow cytometer, make sure that there are no "bubbles" in the membrane filter units for the shut-down solution, cleaning solution, and sheath fluid (0.9% NaCl). "Bleed out" bubbles if there are bubbles in the capsules. Make sure that the waste container is empty, and containers of sheath fluid, water, and 1% bleach in ultrapure water are full.

1. Turn on the flow cytometer and then the computer.
   NOTE: The details of running the flow cytometer explained here are specific to the machine and software demonstrated in the video (see **Table of Materials**). Other software would require appropriate training to use.
2. Open the flow cytometry analysis software, log in to the program, and under the **Cytometer** tab, run **Fluidics Start up**.
3. To create a new experiment, under the **experiment** tab, click **New Folder** and name the folder/experiment appropriately.
4. Click on **new folder** to highlight, then under the **experiment** tab again, click **New Experiment** and name the experiment appropriately.
5. To add the first sample/specimen, under the **experiment** tab, click **New Specimen** and name this specimen

appropriately (name of cell line/control sample/ experiment sample).

6. To add a tube sample, highlight the specimen (group) and under the **experiment** tab, click **New Tube**. Add the appropriate number of tubes and name.

7. To prepare the required graphs, under the **worksheet** tab, open a new worksheet. Once the new worksheet window pops up, open the following using the worksheet screen (hover the mouse across the logo/pictures to find the names):

    1. Prepare a **dot blot** graph of forward scatter (FSC) versus side scatter (SCC) to select the population of interest. Define the first gate by identifying and selecting the population of interest (P1) in a forward and side scatter density plot. Exclude the debris, which constitutes the population with the lowest forward scatter signal.

        NOTE: The FSC parameter detects cells or events based on their size and the SCC discriminates them based on their granularity[20].

    2. Prepare a **dot blot** graph of FSC-area (FSC-A) versus FSC-height (FSC-H) to select the single-cell population. Define the second gate by excluding doublet cell populations, as doublet cells considerably affect the results and conclusions. Exclude doublets by using FSC-H versus FSC-A density plots, where cells of the same size show a similar area and height. Hence, the singlets get clustered diagonally and separated from doublets.

        NOTE: FSC is roughly proportional to the cell size. The voltage pulses are defined as FSC-H, the intensity of the signal, FSC-width that reflects cell size and the duration of the signal, and FSC-A, which is H × W. Doublets have a double width and area value; therefore, gating for singlets is based on detecting disproportions between H, W, and A caused by doublets.

    3. Prepare a **histogram** of the number of events against the fluorophore of interest.

8. Before starting flow cytometry, ensure that the acquisition dashboard for controlling the sample acquisition, inspector, and cytometer to adjust voltage parameters, as well as the worksheet with all the graphs are open.

    NOTE: At least 100 µL of a 10×10^4 cell suspension in a flow cytometry tube is needed to perform the analysis. Especially in case of lower viabilities, propidium iodide staining can be performed to select the viable cell population[21,22].

9. To run the first sample, on the left-hand side of the screen make sure the arrow pointing to the tube is green. If this arrow is not green, click on the arrow to make it green.

10. Using a pipette, transfer each sample from the 96-well black plate to a flow cytometry tube. Run the untreated, unstained control sample on a low speed.

11. On the acquisition dashboard, choose an appropriate number of events to record (30,000), change the **flow rate** to **low**, and click **Acquire Data**.

12. Adjust the voltage for the FSC and SCC parameters. Ensure the cell population is centralized within the dot plot and that no cells are touching either axis of the graph to avoid losing the cells of interest.

13. Increase the acquisition speed to medium or high to analyze the samples faster but do not exceed more than 200 events/s. Then, click **Record Data**.

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14. Perform the gating for P1 (**Figure 4A**) and the single-cell population (**Figure 4B**). Construct the histogram of events against the used fluorochrome and select P1 based on the data (allophycocyanin (APC) in this case) (**Figure 4C**).
15. After adjusting the voltage, gating and recording the data, take out the sample and click **Next Tube**.
16. Insert the next sample, and repeat recording data for all control and test samples (**Figure 3**).
17. Once all the data are collected, wash the flow cytometer by running three tubes of 50% bleach, FACS rinse, and ultrapure water, each for 5 min at a high flow rate.
18. Then, from the **Cytometer** dropdown menu, click **Fluidics Shut Down**.
19. Prior to closing the software and turning off the machine and the computer, export the results as .fcs files to a USB drive to transfer and analyze them, as follows:
    1. In the analysis software, press the **NEW** button to create a new document and window to handle the analysis. Drag the sample files into the new window.
    2. Double-click to open the **unstained sample**. Choose the **P1** population, double-click on the **P1** population to create a **FSC-H versus FSC-A graph**, and gate the single-cell population.
    3. Double-click on the gated single cells to create a histogram of events against the used fluorochrome.
    4. In the original window, select **P1** and **single cells** and drag them to **All Samples** so that all samples now contain the same gating.
    5. Click on the **Layout Editor** button to open the **Layouts** window. Drag two samples (control and test) over one another to create an overlay histogram.

Representative Results

An important aspect of new drug discovery and development is assuring the selectivity and specificity of the drug candidate. This means that the drug candidate should be able to discriminate between different cells and only affect the cell population of interest (selectivity). Selectivity is studied using cell lines that differ in terms of expression of the protein of interest. In this study, MDA-MB-231 and HEK 293T cell lines were chosen as EpCAM-positive and -negative cells. Specificity is another determinant that shows that the protein of interest only responds to a single drug candidate. Here, by using an EpCAM non-binding aptamer, TENN, it was shown that only TEPP attached to EpCAM. In EpCAM-positive cells (MDA-MB-231), overlaying the histograms representing cells treated with TEPP and TENN shows that the TEPP-treated cells are shifted to the right compared to the TENN-treated cells. This shows the binding of the aptamer, TEPP, to the protein of interest, EpCAM (**Figure 4D**). In negative control HEK 293T cells, overlaying histograms of TEPP and TENN does not reflect any shift (**Figure 4E**). This means that in EpCAM-expressing cells, TEPP as the EpCAM aptamer attached to its receptor, and furthermore, no binding was observed in EpCAM-negative cells. These results confirm the selectivity and specificity of the developed aptamer.

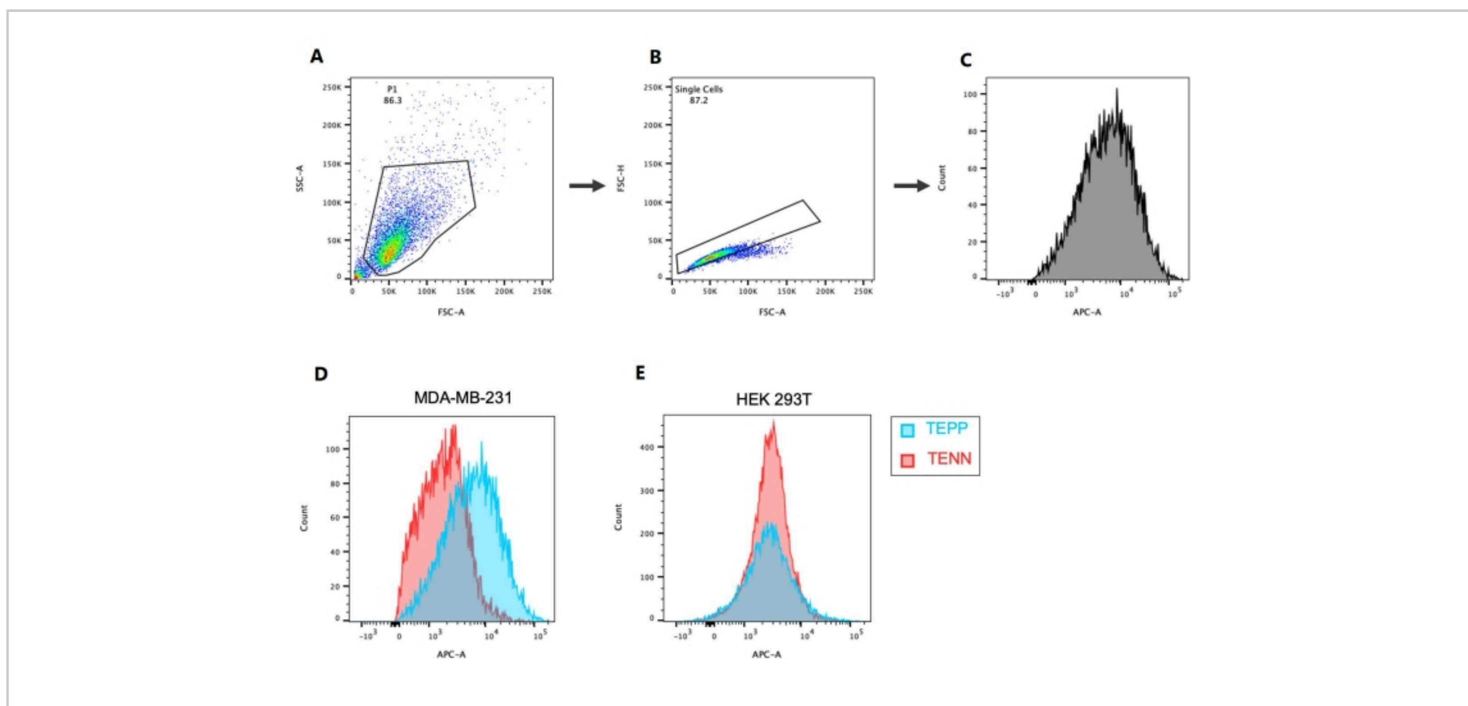

Figure 4: Gating and histograms showing the binding of the cells to the aptamer. (**A**) Selecting the population of cells, (**B**) the single cells, and (**C**) the histogram of the cells attached to the aptamer (200 nM). The binding of EpCAM aptamer (TEPP) versus a non-EpCAM-binding aptamer (TENN) in (**D**) $EpCAM^+$ MDA-MB-231 cells and (**E**) $EpCAM^-$ HEK 293T cells is compared. Abbreviations: EpCAM = epithelial cellular adhesion molecule; FSC-A = forward scatter-peak area; SSC-A = side scatter-peak area; FSC-H = forward scatter-peak height; APC = allophycocyanin. Please click here to view a larger version of this figure.

Discussion

The key challenge with developing new aptamers is the lack of standard guidelines that applies to different steps of this process. McKeague et al. have recently demonstrated some of the associated challenges, which lead to unclear presentations of data in publications and failure to replicate the research. They proposed fundamental guidelines necessary for consideration in characterizing aptamers[19]. An aptamer binding assay is a critical step in screening and/or characterizing aptamers[23], which is widely used by researchers in the field. Since no single guideline exists to display the step-by-step protocol, a flow cytometry method, which is commonly used to study the aptamer-protein binding, is demonstrated in the accompanying video protocol.

There are several methods that measure the interaction of the aptamer and its target. Flow cytometry is one of these methods. Other examples include fluorescence polarization, surface plasmon resonance, capillary electrophoresis, and isothermal titration calorimetry[24]. Choosing the correct method depends on the application of the aptamer. However, it is important to know that each method has its limitations, and that the application of several assays is more beneficial for the characterization of small molecule aptamers[24]. The

method described here has several advantages. It is one of the most reliable, precise, and accurate methods, and is also rapid and cost-effective. The flow cytometric analysis of aptamer binding can be applied in various steps of aptamer development, including candidate screening, truncation and optimization, characterization, and validation.

With fluorescence labeling, there are choices to either label or use the intrinsic fluorescence of the target or label the aptamer[24]. In the authors' experience, using labeled aptamers is reliable and easy to set up. Aptamers can be labeled with a fluorophore at any end (3' or 5')[25]; however, the end with less guanine is more favorable, as guanine can quench the fluorescence and its detection[26]. A disadvantage of this method is that the aptamer should be tagged to a fluorophore. Furthermore, although this method reflects the binding of the aptamer to the specific protein, it does not display the location of the interaction site. Hence, further studies, such as fluorescence microscopy, might be required to confirm the aptamer-protein interaction location[25].

There are some critical notes to be considered while performing this assay. It is important to consider that each aptamer has specific requirements for handling and folding. This includes the choice of reconstitution and dilution buffers and the folding conditions. Reconstitution of lyophilized aptamers occurs in purified sterile pyrogen- and RNase-free ultrapure water. This is to minimize the concentration of ions around the nucleotides. Ion concentration highly affects the formation of 2D structures of aptamers and their affinity and stability. Hence, for further reconstitution of the aptamer stock, care should be taken in the correct preparation of other buffers and metal cationic solutions, such as $MgCl_2$[25,27]. The optimum concentration of metal cationic solution shields the negative charge of the aptamer yet, does not inhibit the interaction of the aptamer with its target.

Furthermore, because the aptamer has a potential for a non-specific, off-target binding, the application of blocking buffer plays a critical role in the binding assay. In addition to the negatively charged BSA, which is commonly used for antibodies, salmon sperm DNA or tRNA is also required here. This mixture blocks the positively charged proteins and nucleic acid binding sites. This blocking stage is specifically important for the selectivity and specificity of aptamers towards cancer cells, due to their negative charge compared to neutral and positively charged normal cells[28]. Furthermore, the 3D folding of aptamers is dependent on other factors such as temperature. The importance of conditions affecting the 3D formation of the aptamer, including the choice of tube and the duration of PCR phases, has been reviewed[25]. Moreover, the incubation time of the aptamer with the target should be optimized for every specific aptamer. The incubation temperature is also highly important. Maintenance of a temperature of 4 °C is especially critical for cell surface proteins that can easily be internalized, such as EpCAM[25].

The other important factor in this assay is the application of proper controls. Cancer cells that either express or do not express the target protein should be used to evaluate the selectivity of the aptamer. In each experiment, in addition to the aptamer of interest, a negative control aptamer (a random sequence or a scrambled sequence) is required to show the specificity of the aptamer. Ideally, this control should have a similar length of nucleotides and undergo similar folding as the aptamer. In this experiment, a negative control (TENN), an aptamer with a similar structure to TEPP shown to have a low binding affinity with EpCAM, was used[10].

The protocol presented here is a qualitative assay and can be further used for the quantitative assessment of binding affinity and the determination of the dissociation constant (K_d)[29,30,31]. However, it is important to show the reproducibility of the results using technical and biological replicates. To achieve this, it is highly important to consider major determinants to properly perform this assay. This could include, but is not limited to, using a low passage number of mycoplasma-free cells that are properly grown in their optimal conditions, using a constant number of cells to be exposed with the aptamer in each replicate, the application of proper temperature and folding conditions, maintaining similar experimental conditions for both the aptamer and the control, maintaining minimal exposure of the aptamers to environmental light, using optimized aptamer-cell exposure time and temperature, maintaining a temperature of 4 °C for the cells, which includes precooling the centrifuge, the 96-well plate, and the flow cytometry tubes, and accurately preparing the buffers, with an ionic concentration as close to the claimed concentrations as possible.

Disclosures

The authors have no conflicts of interest to disclose.

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