First of its Kind!

Fluorescent Universal Lateral Flow Assay Kit

for Detection of Nucleic Acids.

Introduction:

Lateral flow assays have traditionally relied on the use of reagents that have been attached to colored detection moieties such as gold nanoparticles. Colorimetric readouts allow rapid visual assessment of the assay result. However, despite the extensive and well-documented use of colorimetric detection methods, many researchers are instead turning to fluorescent labels since they have been demonstrated to improve LF test strip sensitivity by at least 10-fold and as much as 300-fold!

We have screened numerous conditions for generating conjugates between functionalized oligonucleotides with fluorescent particles to find the optimized (1) association, (2) ratio of fluorescent particles to functionalized oligonucleotides, (3) component concentrations, and (4) blocking conditions. In addition, we have screened numerous types of fluorescent particles to find the most appropriate conjugate spraying and drying conditions, conjugate pad materials, buffers, and stability. Collectively, these investigations have given us an enormous wealth of information on how to effectively convert a colloidal gold based LFA device to a stable, robust, and extremely sensitive fluorescent based LFA.

To address a key market need, Attogene has now launched the first of its kind fluorescent particle based Fluorescent Universal Lateral Flow Kit for the development of nucleic acid based lateral flow assays. This kit pushes the boundaries of conventional gold-based LFA test kits and creates a realm of higher sensitivity tests ranging from a projected 10-300-fold greater sensitivity than those using colloidal gold particles. Attogene has developed these key materials to support this growing field using our internal lateral flow manufacturing equipment and ingenuity. The novel strategic ingredients of our kit include specialized highly stable fluorescent particles that are embedded into conjugate pads and applied to cards containing nitrocellulose adaptable test and control lines. These fluorescent particles are extremely stable and can be visualized using a standard black light or with commercial fluorescent lateral flow readers.

In the nucleic acid detection study outlined below we characterize a fluorescent particle streptavidin conjugate to oligonucleotides containing biotin, fluorescein (FAM) or digoxigenin (Dig).

Results:

Research efforts exploring fluorescent particles in lateral flow-based tests have ushered an exciting new era of advancement in increasing the sensitivity of lateral flow tests. Reports have estimated that fluorescent particles can enhance sensitivity of the lateral flow detection limits 10 to 300-fold. Our fluorescent universal lateral flow assay kits enable all users to harness this fluorescent particle revolution, including those users that are limited by the need for expensive instrumentation which is used for spraying nitrocellulose membranes with biological materials and evenly cutting cards into strips. In addition, Attogene provides tests with validated and optimized components to enhance users' exploration of the field of fluorescent lateral flow assay development. Attogene in the past has released numerous standardized materials including colloidal gold, colloidal gold conjugates, cassettes, and critical lateral flow assay running buffers. Now, we are excited to have recently released the first of its kind fluorescent particle based Fluorescent Universal Lateral Flow Assay Kit (AU2037). This kit contains strips composed of highly stable, optimized streptavidin conjugated fluorescent particles that are embedded in conjugate

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pads attached to cards containing a nitrocellulose membrane sprayed with biotin, anti-FAM and anti-Dig capture lines (custom configurations are also available upon request).

To demonstrate the accuracy and utility of our kit, dipsticks that are part of our fluorescent Universal Lateral Flow Assay Kit were used in a stochiometric study with a synthetic nucleic acid containing 5' biotin and 3' FAM moieties. To analyze stochiometric effects, the biotin and FAM labeled nucleic acids were mixed at increasing concentrations with 150µL of a proprietary specially designed lateral flow running buffer in a well of a 96 well plate or applied to our standard cassettes Lateral Flow Cassettes.

Lane Number	Fluorescent Particle Concentration
1.	1000 fmol
2.	500 fmol
3.	250 fmol
4.	125 fmol
5.	62.4 fmol
6.	31.2 fmol
7.	15.6 fmol
8.	7.8 fmol
9.	3.9 fmol
10.	1.94 fmol
11.	0.96 fmol
12.	0.48 fmol

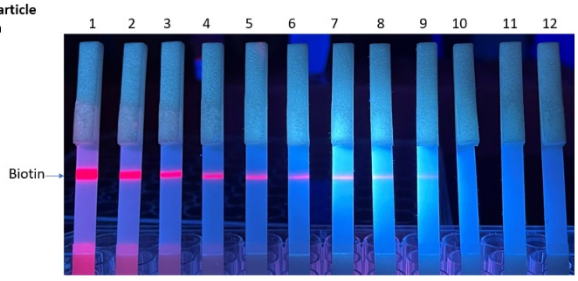


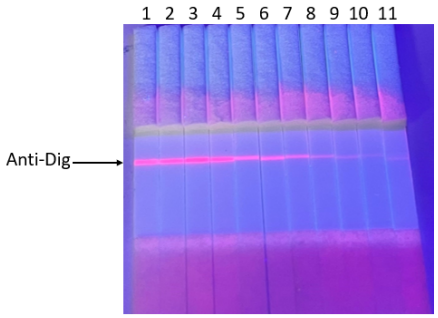
Figure 1. Detection limit analysis of streptavidin fluorescent particles on strips containing a sprayed biotin. The strips were run by mixing the Fluorescent Lateral Flow Assay Running Buffer with limiting amounts of streptavidin conjugated fluorescent particles. After 15 minutes, the strips were visualized using a black flashlight in the dark and a picture was taken using a cell phone camera. Signal was easily apparent by eye using as low as 3.9 fmoles of particles.

Because not every laboratory has a fluorescent lateral flow assay reader – and to demonstrate how simple a readout from these assays can be, the data shown in Figures 1-4 demonstrate the importance of the relative amounts of streptavidin conjugated fluorescent particles needed to uncover optimal line intensities using a standard black light flashlight and inspection using a cell phone camera. The biotin sprayed line captures streptavidin conjugated fluorescent particles when streptavidin in not occupied with the dual labeled oligonucleotide. **Figure 1** shows that as little as roughly 3.9 fmoles of the streptavidin conjugated fluorescent particles used in our fluorescent universal lateral flow assay kit can be easily detected by eye after illumination with a black light. Use of a fluorescent LFA reader to analyze the data increases the sensitivity by at least another log as one would anticipate (data not shown).

We next performed an experiment of limiting dilution of an oligonucleotide that contained biotin and DIG while keeping a constant amount of streptavidin fluorescent particles. What these data (**Figure 2**) demonstrate is that oligonucleotide concentrations can be readily detected by eye using a black light at 19 femtomoles and maximal binding is detected at 1.26-0.625 picomoles of oligonucleotide. When using a fluorescent particle lateral flow reader, the limit of detection is, as expected, once again significantly

Figure 2. Streptavidin conjugated fluorescent particles were incubated with varying concentrations of an oligonucleotide containing a biotin and Dig at the 5' and 3' ends respectively. The strips containing anti dig antibody were run by mixing with the Fluorescent Lateral Flow Assay Running Buffer. After 15 minutes, the strips were visualized using a black light in the dark and a picture was taken using a cell phone camera. Signal was easily apparent by eye using as low as 19 fmoles of oligonucleotides per well.

Lane Number	Amount of Biotin-Oligo-Dig
1.	5 pmol
2.	2.5 pmol
3.	1.25 pmol
4.	0.625 pmol
5.	0.312 pmol
6.	0.156 pmol
7.	0.078 pmol
8.	0.039 pmol
9.	0.019 pmol
10.	0.009 pmol
11.	0.0045 pmol



lower (data not shown). We also observed that roughly 10 picomoles of oligonucleotide was enough to completely saturate all the streptavidin binding sites on the strep conjugated fluorescent particles. Each well contained 150µL of our proprietary fluorescent Nucleic Acid Universal Strip Running Buffer, 1 picomole of streptavidin fluorescent particle, and varying amounts of biotin labeled oligonucleotide. We find that at between 10-20 picomoles of biotinylated oligonucleotide with 1 pmole of particles is the ratio where saturation of the particles is observed. This equated to a roughly 1:10 ratio of particle to oligo. This would make sense for particles containing more than one streptavidin per particle as each streptavidin can associate with more than one biotin. Interestingly, we have detected different saturations using different oligonucleotides indicating the possibility for structural features having an impact on accessibility and binding capability to the beads. This should be considered when comparing the ratios of your specific molecules.

Next, we tested for the capability of a biotin labeled oligonucleotide to saturate the streptavidin binding sites on the fluorescent nanoparticles **(Figure 3)**. To do this test, we kept the concentration of the fluorescent particles constant and varied (from high to low) concentrations of biotin labeled oligonucleotide. Starting from 150pmoles we completely saturated to beads while a sequential reduction in biotin oligonucleotide concentration reveals that between 10-20 pmoles is the inflection point in which fully saturating the biotin sites on the beads with oligonucleotides inhibits their ability to bind to biotin on the strip.

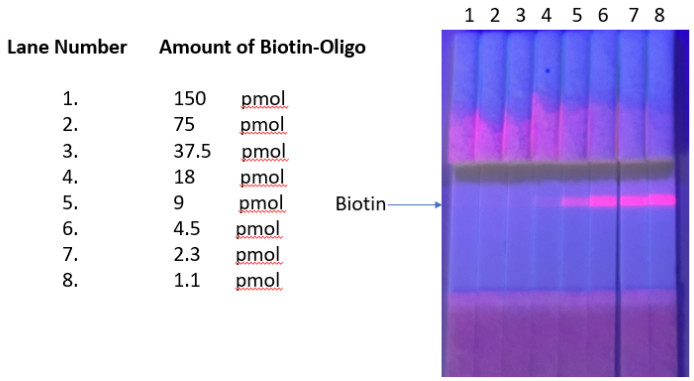


Figure 3 Each well contained 150µL of our proprietary fluorescent nucleic acid universal strip running buffer, 1 pmole of strep fluorescent particle and varying amounts of biotin labeled oligonucleotide. We find that at between 10-20 pmoles of biotinylated oligonucleotide with 1pmole of particles is the ratio where saturation of the particles is observed.

Finally, we analyzed the performance of a dual labeled oligonucleotide containing a FAM on the 5' end and a biotin on the 3' end in the assay **(Figure 4)**. One picomole of streptavidin-conjugated fluorescent particles either lacking (Lane 1) or containing varying amounts of an oligonucleotide with biotin and FAM (Lane 2) were mixed with our proprietary Universal Lateral Flow Assay running buffer. The data shown in **Figure 4** demonstrates the specificity of our test and the robustness of detection using a black light in panel A or using a fluorescent LFA detector system (panel B).

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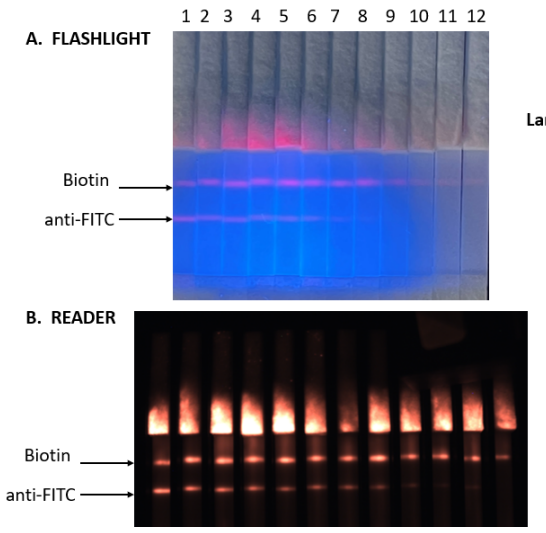

Lane Number	Amount of Biotin-Oligo
1.	20 pmol
2.	10 pmol
3.	5 pmol
4.	2.5 pmol
5.	1.25 pmol
6.	0.625 pmol
7.	0.3125 pmol
8.	0.156 pmol
9.	0.7812 pmol
10.	0.039 pmoles
11.	0.0195 pmoles
12.	No FITC – oligo - biotin

Figure 4. Lateral flow strips sprayed with biotin and anti-FITC of streptavidin conjugated fluorescent particles either lacking (Lane 12) or containing a specific amount of picomoles of an oligonucleotide with biotin and FITC (Lane 1-11) were mixed with our proprietary universal LFA running buffer. These data demonstrate the specificity of our test and the robustness of detection using a black light in Panel A or using a fluorescent LFA detector system in Panel B.

Conclusions:

As observed with colloidal gold based universal lateral flow assays, the stoichiometry of reagent binding sites in these fluorescence-based lateral flow assay is important to balance for optimal performance. Ratio adjustments are often needed for careful adjustments to optimize the intensity of the readout lines. We have noted that oligonucleotides of different length and sequence can give different binding results. Therefore, these oligomer characteristics represent an important factor for understanding binding affinity. Linkers that provide distances between the biotin (or other readout) molecule and the nucleic acid can also be an influencing feature. Lastly, these items in combination with running buffer composition can provide an additional variable to optimize to make your test as sensitive as possible.

Attogene is always here to help you with your assay optimization needs, feel free to email us at sales@attogene.com for additional details.