

Development of a microwell-based lateral flow immunoassay for detection of tetracycline in cow milk

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Abstract

In order to meet the increasing demand for dairy products in China big cattle farms have been formed throughout the country. To control the outbreak and spread of disease antibiotics are readily employed, and detection methods are needed to ensure that the produced milk does not contain levels of antibiotics exceeding the regulatory limits. The aim of this project was to develop and optimize a commercially utilizable microwell-based lateral flow immunoassay for the detection of the antibiotic tetracycline in cow milk. The final conditions for the developed test strip included a test line with an antigen (TC-BSA) concentration of $2\mu\text{g}/\mu\text{l}$ and a control line with a secondary antibody (goat anti-mouse IgG) concentration of $0.08\mu\text{g}/\mu\text{l}$. Each microwell contained $5\mu\text{l}$ of detection antibody (anti-TC) with a concentration of $0.12\mu\text{g}/\mu\text{l}$ as well as $5\mu\text{l}$ of Tris-HCl buffer with pH 7.5 and $5\mu\text{l}$ of the surfactant Tetronic 1307. These conditions resulted in an assay detection limit of 30ppb tetracycline and an expected shelf life of at least 12 months, with a total assay time of around 6-7 minutes.

Table of contents

1. Introduction	4
1.1 Aim	4
1.2 Tetracycline	4
1.3 Lateral flow immunoassay	5
1.3.1 Components	6
2. Materials and Method	8
2.1 Preparation of colloidal gold	8
2.2 Optimization of antibody-gold conjugation	8
2.3 Preparation of detection antibody	8
2.3.1 10% BSA	9
2.3.2 Redissolution solution	9
2.4 Optimization of antigen concentration	9
2.4.1 Phosphate buffer	10
2.5 Optimization of detection antibody concentration	10
2.6 Optimization of detection antibody volume	10
2.7 Optimization of pH in microwell	11
2.7.1 Tris-HCl buffers of varying pH	11
2.8 Optimization of surfactant in microwell	11
2.8.1 10% Tween 20	12
2.8.2 5% Tetronic 1307 and BAC	12
2.9 Determination of test strip detection limit	12
2.10 Formation of control line	13
2.11 Freeze-drying of microwell content	13
2.12 Accelerated stability testing	14
3. Results	15
3.1 Characterization of colloidal gold and antibody-gold conjugation	15
3.2 Optimal antigen concentration	17
3.3 Optimal detection antibody concentration	18
3.4 Optimal detection antibody volume	19
3.5 Optimal pH in microwell	20
3.6 Optimal surfactant in microwell	21
3.7 Test strip detection limit	23
3.8 Control line	23
3.9 Finished product	27
3.10 Accelerated stability testing	28
4. Discussion	30
4.1 Future work	31
References	33

1. Introduction

The last few decades have seen a substantial increase in the demand for dairies in China, and this has in turn shifted the dairy production of the country from small-scale, local farms to industrialized mega-farms. One of the major challenges that such a mega-farm faces is the maintaining of animal welfare since the number of cattle residing in a very restricted area can reach numbers up to ten thousand (Balch 2014). The most efficient way to control and prevent the outbreak of disease is through treatment with different kinds of antibiotics, many of which are readily available without any kind of prescription throughout China (McLaughlin 2016).

Residues of antibiotics present in dairy products can have negative effects on human health and is a matter of food safety. In addition to this, uncontrolled and irresponsible over-usage of antibiotics in the livestock sector has been shown to increase bacterial resistance against these drugs (Granados-Chinchilla et al. 2017). This has severe implications for the future health prospects for people all around the globe, as common diseases and small injuries might lead to untreatable, life-threatening conditions. Based on this there is an undeniable need for simple yet trustworthy methods capable of detecting the levels of antibiotics present in milk, to ensure that precautionary regulations set by the government are followed.

1.1 Aim

The aim of this project was to develop and optimize a commercially utilizable microwell-based lateral flow immunoassay for the detection of the antibiotic tetracycline in cow milk. This included finding optimal antibody and antigen concentrations, pH values and choice of surfactant for construction of a highly sensitive yet easily assembled assay.

1.2 Tetracycline

Tetracyclines (TCs) are a group of broad-spectrum antibiotics that are commonly used in cases of veterinary medicine. Their effect on bacteria is a result of TCs blocking the ribonucleic acid aminoacyl from binding to the ribosome, thus obstructing the bacterial protein synthesis. If ingested with food TCs might, in addition to increase the antibiotic resistance of bacteria, also cause more direct harm in the body such as allergic reactions, deformation of mucus tissues and problems with the gastrointestinal tract. The most commonly used antibiotics from the tetracycline family used in veterinary cases are tetracycline (TC),

chlortetracycline (CTC) and oxytetracycline (OTC) (Taranova et al. 2015). The EU requirement for tetracycline in milk is a maximum residue limit (MRL) of 100µg/kg (100ppb) while the Chinese government standard is an MRL of 50µg/kg (50ppb) (Fang and Zweigenbaum, n.d.).

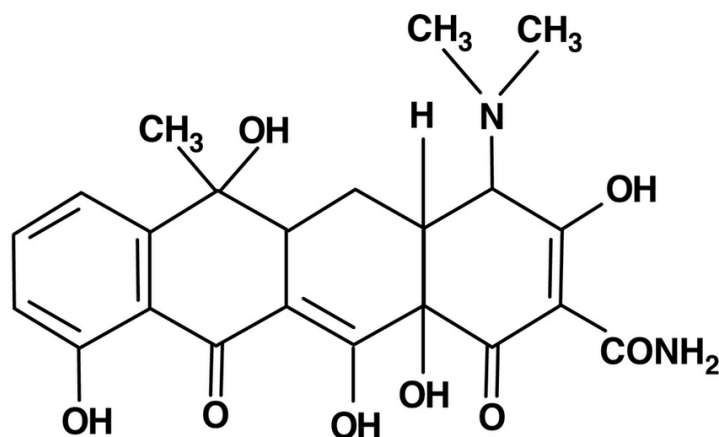


Figure 1. Showing the molecular structure of tetracycline (ResearchGate 2017)

1.3 Lateral flow immunoassay

Also known as immunochromatographic test paper technology, the lateral flow immunoassay (LFIA) allows rapid detection of specific analytes in a sample with the help of immunological techniques (Shan et al. 2014). The first commercially successful product that applied the LFIA technology was the Clearview home pregnancy test, which was launched in 1988 by Unipath. Since then the use of LFIA has spread to numerous other fields and is today used in for instance clinical trials, the agricultural sector and the food industry (Cytodiagnosics 2017).

The main principle behind LFIA is the migration of a sample through a test strip upon which biological components with binding capacity to the target analyte have been pre-immobilized. Two different assay formats are used depending on the properties of the analyte; the sandwich assay and the competitive assay. The sandwich approach is suitable for bigger analyte molecules that can be bound to two antibodies simultaneously whereas the competitive approach is preferred when the analyte is relatively small and only capable of binding one antibody (Shan et al. 2014). For detection of tetracycline in cow milk the competitive version is therefore best suited.

One of the main strong points of LFIA is its simple arrangement that enables detection tests to be performed directly on site without the need to transport samples to a laboratory (Tecnalab 2017). This, in combination with uncompromised

sensitivity, low production costs and quickly obtained results, has rightfully brought LFI to the frontline of immunologic analytical methods.

Figure 2 shows the overall principle for a competitive lateral flow immunoassay.

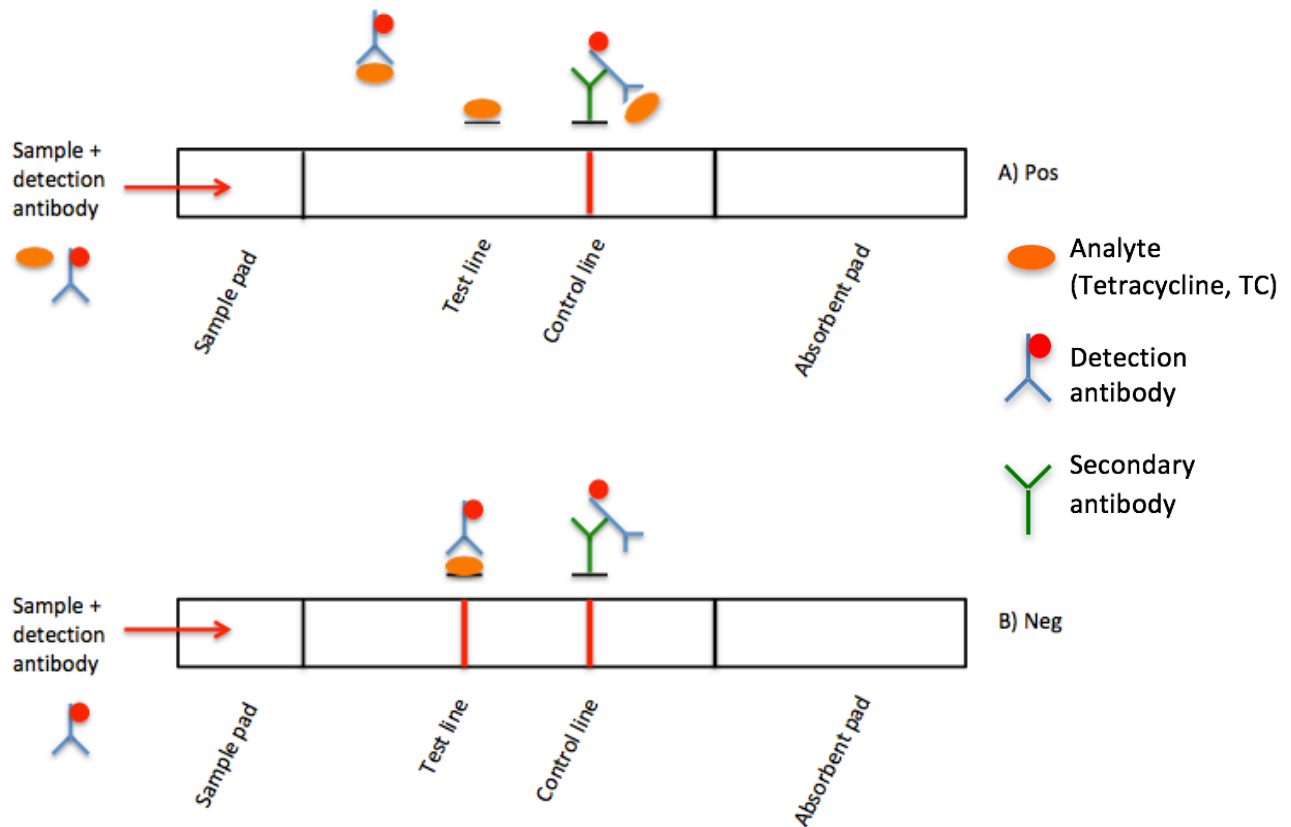


Figure 2. Showing the principle behind competitive lateral flow immunoassay. A) *Positive result*. Since the analyte (TC) is present in the sample it forms complexes together with the detection antibody, and so when the sample passes the TC present on the test line no or little detection antibody will be free to bind causing the test line to remain blank or only vaguely colored. B) *Negative result*. The analyte is not present in the sample leading to the detection antibody binding to the TC present on the test line, giving rise to a prominent red line. In both positive and negative cases the secondary antibody on the control line will bind the detection antibody to ensure proper functioning of the test. (Adapted from *Guide to Lateral Flow Immunoassays* by Innova Biosciences, 2016).

1.3.1 Components

Conventional LFIA test strips consist of a sample pad, a conjugate pad, an absorbent pad and a nitrocellulose membrane, all attached to some sort of supporting material for increased stability (Hsieh et al. 2017).

The main function of the **sample pad** is to absorb the sample added and to further distribute it along the test strip in a controlled manner. It is important that the

material has a low protein-binding ability to prevent any loss of analyte (Innova Biosciences 2017).

In the most common version of lateral flow immunoassays a conjugate pad is situated right next to the sample pad. The conjugate pad has the detection antibody sprayed and dried onto it, which is released into the liquid and starts reacting with the analyte as the sample passes by. There are however studies having shown that by using **microwells** containing freeze-dried detection antibodies the sensitivity of the assay is further increased (Han et al. 2016). The function of the microwells is thus to contain and preserve the detection antibody in conditions favorable for the assay, obtained by addition of buffers for maintaining certain pH values or surfactants promoting uniform movement of the sample along the strip membrane (Taranova et al. 2015). In addition to this the microwells serve as the platform for the assay itself since the milk sample is simply added into the them and mixed with the detection antibody, followed by the insertion of the test strip.

The **nitrocellulose (NC) membrane** incorporates the very core of the lateral flow immunoassay; the test and control lines. The test line consists of capture molecules that bind the target analyte while the control line contains secondary antibodies specific for the detection antibody. The control line consequently validates that the assay is working properly. The attachment of the capture molecules to the membrane requires instrumentation and most commonly positive displacement systems are used. The principle behind positive displacement is to directly dispense the liquid in a line on to the membrane. After the liquid dispensing it is essential that the membrane is allowed to dry completely to ensure sufficient affixation of the capture molecules (Innova Biosciences 2017).

At the end of the lateral flow immunoassay is the **absorbent pad**. It is necessary for increasing the sample volume taken up by the test strip as well as preventing backflow of the liquid. Increased sample volume helps to wash away any unattached detection antibodies and therefore minimizes the background interference, improving the assay sensitivity (Innova Biosciences 2017).

The **detection antibody** is an antibody specific for the analyte that has been conjugated with the **detection moiety**, often gold nanoparticles which produce colored readouts. Monoclonal antibodies are preferred over polyclonal ones as they can be produced in large quantities while maintaining high specificity since they recognize a single epitope. The stability of the detection antibody is a key factor for the assay since it needs to maintain its characteristics even after having been dried or dry-frozen (Innova Biosciences 2017).

2. Materials and Method

2.1 Preparation of colloidal gold

The colloidal gold was prepared using the Frens (Turkevich) method (Wang et al. 2014). 100ml of 0.01% (w/v) chloroauric acid was added into a 250ml Erlenmeyer flask and heated up to its boiling point, after which 2ml of 1.0% (w/v) sodium citrate was added. The yellow solution was allowed to boil while being continuously stirred for approximately five minutes, until the color had changed to red. The solution was then cooled down by continued stirring at room temperature and eventually stored at 4°C. 100µl of the solution was transferred into an ELISA strip plate microwell (JetBIOFIL) in order to read off the optical density at wavelengths between 400 and 800nm. The newly formed colloidal gold was then examined with a transmission electron microscope.

2.2 Optimization of antibody-gold conjugation

1ml of colloidal gold solution was added into each of seven different Eppendorf tubes. To find the optimal alkalinity for the conjugation of the gold nanoparticles to the detection antibody 0, 6, 8, 10, 12, 14 and 16µl of 0.2M K₂CO₃ (potassium carbonate) was added respectively into the Eppendorf tubes, followed by 8µg of the detection antibody (monoclonal anti-TC, mouse IgG from Guangzhou Ucando Biotechnology, 6µg/µl). The Eppendorf tubes were left to incubate for 15 minutes and then 100µl of each solution was transferred into microwells and the optical density was read off at a wavelength of 530nm (found in step 2.1 to be the wavelength at which the prepared colloidal gold solution had the highest optical density). The amount of added K₂CO₃ that resulted in the highest optical density at 530nm was considered the optimal amount to use (Wang et al. 2012).

2.3 Preparation of detection antibody

1ml of colloidal gold solution was added into an Eppendorf tube, followed by the addition of 8µl (found to be the optimal amount in step 2.2) of 0.2M K₂CO₃. 8µg of the detection antibody was added and the tube was left to incubate for 15 minutes followed by the addition of 100µl of 10% BSA (see 2.3.1) and another 15 minutes of incubation time. The tube was then centrifuged at 12000rpm for 15 minutes. The supernatant was removed, leaving only the pellet of antibody gold conjugates at the bottom which were thereafter re-dissolved in 100µl of redissolution solution (see 2.3.2). This resulted in a detection antibody concentration of 0.08µg/µl.

2.3.1 10% BSA

50g of BSA (Bovine Serum Albumin) powder was weighed up and dissolved in 500ml of distilled water in a 500ml Erlenmeyer flask. The solution was then stored in aliquots of 50ml in -20°C .

2.3.2 Redissolution solution

5g of sucrose, 2.5g of mycose and 0.5g of BSA was dissolved in 50ml of distilled water, followed by the addition of 0.5ml Tris buffer of pH 8.0. The solution was then stored in -20°C .

2.4 Optimization of antigen concentration

The antigen (TC-BSA conjugates from Guangzhou Ucando Biotechnology, $15\mu\text{g}/\mu\text{l}$), was prepared in small PCR-tubes. The concentrations 1, 2, 3 and $4\mu\text{g}/\mu\text{l}$ were prepared by dilution with 10mM phosphate buffer (see 2.4.1), each concentration being prepared in amounts of $10\mu\text{l}$. The different antigen concentrations were then attached to NC membranes (Unisart CN 140, Sartorius) to form test lines using a contact dispenser (XYZ dispenser HM3030, Kinbio). The dispensing settings were set to a rate of 10cm/s with a dispensing quantity of $0.8\mu\text{l}$ antigen/cm. The NC membranes were then dried at 55°C for 15 minutes. The full test strips were then assembled by attaching the NC membranes, absorbent pads and sample pads to the supporting back material followed by cutting it into strips using Automatic Strip Cutter ZQ2000 from Kinbio. The width of each test strip was set to 0.4 cm.

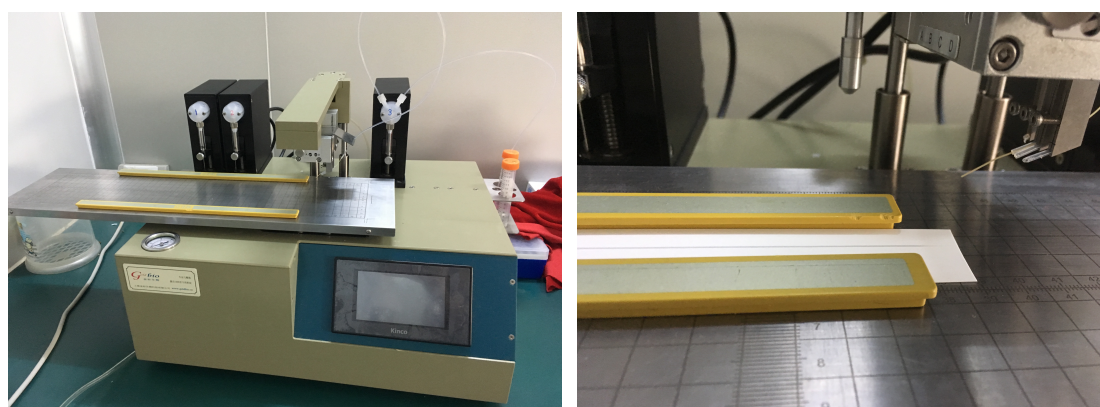


Figure 3. Showing the contact dispenser (left) and the nitrocellulose membrane with recently attached antigen (right).

200µl of pasteurized, whole cow milk from the brand 香满楼 was added into each of eight microwells. 5µl of the detection antibody prepared in step 2.3 was then added to each microwell followed by mixing by pipetting up and down a few times. The test strips were then inserted into the microwells, two of each antigen concentration. The test strips were left in the microwells for three minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test lines. The antigen concentration that was determined optimal was then used for production of more test strips for further use in following steps.

2.4.1 Phosphate buffer

Two different solutions (A and B) were prepared separately. For solution A 27,6g of monosodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) was dissolved in 1000ml of deionized water. Solution B was prepared by dissolving 53,6g of disodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$) in 1000ml of deionized water. 39ml of solution A and 61ml of solution B were then mixed together in a separate tube, forming a phosphate buffer stock solution of 200mM and pH7, stored at 4°C. Before usage it was further diluted to 10mM.

2.5 Optimization of detection antibody concentration

Using the method described in step 2.3 but changing the amount of added antibody to 6, 9, 12 and 15µg, four different detection antibody solutions were prepared with the final concentrations of approximately 0.06, 0.00, 0.12 and 0.15µg/µl.

200µl cow milk was added into eight different microwells. Each detection antibody concentration was added in duplicates into the microwells in amounts of 5µl, followed by mixing through pipetting up and down a few times. The test strips were then inserted into the microwells. The test strips were left in the microwells for three minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test lines.

2.6 Optimization of detection antibody volume

200µl cow milk was added into eight different microwells. 5, 10, 15 and 20µl of the detection antibody solution with the optimal concentration as found in step 2.5 (0.12µg/µl) was added into each of the microwells, all in duplicates. This was

followed by mixing through pipetting up and down a few times. The test strips were then inserted into the microwells. The test strips were left in the microwells for three minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test lines.

2.7 Optimization of pH in microwell

200 μ l cow milk was added into ten different microwells. 5 μ l of Tris-HCl buffer with pH values 7.5, 8.0, 8.5 and 9.0 respectively were added into the microwells, all in duplicates. In two control microwells no Tris-HCl buffer was added. In accordance with the optimal detection antibody concentration and volume found in steps 2.5 and 2.6 respectively, 5 μ l of 0.12 μ g/ μ l detection antibody was added into each microwell followed by mixing through pipetting up and down a few times. The test strips were then inserted into the microwells. The test strips were left in the microwells for three minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test lines.

2.7.1 Tris-HCl buffers of varying pH

A 1L measuring cylinder was filled with 800ml of distilled water. 121,1g of Tris base was weighed up using a scale and added to the water, followed by the insertion of a pH-meter and addition of 1M HCl (hydrochloric acid) until the desired pH was reached. Distilled water was then added until the 1L mark.

2.8 Optimization of surfactant in microwell

200 μ l cow milk was added into eight different microwells. 5 μ l of Tris-HCl buffer with pH 7.5 (determined to be the optimal pH in step 2.7) was added into each microwell. 5, 10 and 15 μ l of 10% (v/v) nonionic surfactant Tween 20 (polyoxyethylene 20 sorbitan monolaurate) was added into the microwells, each different volume in duplicates. In two control microwells no surfactant was added. In accordance with the optimal detection antibody concentration and volume found in steps 2.5 and 2.6 respectively, 5 μ l of 0.12 μ g/ μ l detection antibody was added into each microwell followed by mixing through pipetting up and down a few times. The test strips were then inserted into the microwells. The test strips were left in the microwells for three minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test lines.

The procedure was repeated with 5% (w/v) amphoteric surfactant Tetronic 1307 (ethylenediamine alkoxlate block copolymer) and 5% (w/v) cationic surfactant BAC (benzalkonium chloride) respectively.

2.8.1 10% Tween 20

5g of Tween 20 liquid (SIGMA-ALDRICH) was weighed up and added into a 50ml Erlenmeyer flask followed by the addition of distilled water until the 50ml mark. The solution was mixed and distributed into aliquots of 1ml, stored at 4°C.

2.8.2 5% Tetronic 1307 and BAC

2,5g of Tetronic 1307 powder (BASF) was weighed up and added into a 50ml Erlenmeyer flask followed by the addition of distilled water until the 50ml mark. The solution was mixed and distributed into aliquots of 1ml, stored at 4°C.

This was repeated with BAC powder (SIGMA-ALDRICH).

2.9 Determination of test strip detection limit

A tetracycline stock solution with concentration 1000mg/L (1000ppm) was bought from 中国药品生物制品检定所 (China's National Institute for Food and Drug Control). This was used to prepare 1ml of tetracycline diluted in cow milk with the concentration 120ppb. 400µl of the 120ppb tetracycline solution was added into a microwell and 200µl of cow milk was added into five different microwells. 200µl of the 120ppb solution was then transferred into the first of these microwells and the content carefully mixed by pipetting up and down eight times. The process of transferring 200µl of solution to the next microwell was repeated until the last microwell, in which nothing was added and the remaining solution was discarded. This resulted in six microwells containing 200µl of cow milk with the following tetracycline concentrations: 120, 60, 30, 15, 7.5 and 0ppb. The procedure was repeated so that each concentration was available in duplicates.

5µl of Tris-HCl buffer with pH 7.5 (determined to be the optimal pH in step 2.6) microwell and 5µl of Tetronic 1307 (determined to be the optimal surfactant and volume in step 2.8) was added into the microwells. In accordance with the optimal detection antibody concentration and volume found in steps 2.5 and 2.6 respectively, 5µl of 0.12µg/µl detection antibody was added into each microwell followed by mixing through pipetting up and down a few times. After three minutes (to allow the binding of tetracycline to detection antibodies in the microwells) the test strips were inserted. The test strips were left in the microwells for another three

minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test lines.

2.10 Formation of control line

The secondary antibody used for the control line was goat anti-mouse IgG (13mg/ml, Berseebio). 10 μ l of the secondary antibody was prepared in a small PCR-tube in the concentration 0.25 μ g/ μ l by dilution with 10mM phosphate buffer. 10 μ l of the antigen (TC-BSA conjugates) used for the test line was prepared in another PCR-tube in the concentration 2 μ g/ μ l (chosen to be the optimal concentration in step 2.4). The two solutions were attached to an NC membrane, the control line located 8mm over the test line, and full test strips were assembled according to the method described in step 2.4.

The 1000mg/ml tetracycline stock solution used in step 2.9 was further used to prepare four times 1ml of cow milk containing tetracycline in the concentrations 0, 15, 30 and 45ppb. 200 μ l of the different concentrations, each in duplicates, were added into microwells. This was followed by the addition of 5 μ l of pH 7.5 Tris-HCl buffer, 5 μ l of 5% Tetronic 1307 and 5 μ l of the detection antibody with concentration 12 μ g/ μ l, all according to previously determined optimal conditions. After three minutes the newly produced test strips were inserted into the microwells and left there for another three minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test- and control lines.

This process was then repeated for two additional concentrations of the secondary antibody: 0.15 μ g/ μ l and 0.08 μ g/ μ l. The optimal concentration of the secondary antibody was then used for large-scale production of test strips.

2.11 Freeze-drying of microwell content

For the freeze-drying each microwell was to contain a total amount of 100 μ l liquid, consisting of 5 μ l pH 7.5 Tris-HCl buffer, 5 μ l 5% Tetronic 1307, 5 μ l of detection antibody with the concentration 12 μ g/ μ l, 50 μ l stabilizing buffer and 24,5 μ l distilled water. This was prepared for large-scale production by multiplying every amount with 700, resulting in 24.5ml distilled water, 35ml stabilizing buffer, 3.5ml pH 7.5 Tris-HCl buffer, 3.5ml 5% Tetronic 1307 and 3.5ml of detection antibody with the concentration 12 μ g/ μ l being added into a 100ml Erlenmeyer flask. 672 microwells were then filled with 100 μ l of the liquid and put into a freeze-dryer (EPSILON 2-8D,

Christ) for freeze drying following a specific cycle developed by the lab. The finished product was then packed together with produced test strips in plastic bottles with desiccant caps for protection against moisture. Each plastic bottle was filled with eight microwells and eight test strips.



Figure 4. Showing the microwells right before the freeze-drying process.

2.12 Accelerated stability testing

Four desiccant bottles containing the finished product (eight sets of microwells and test strips) were put into an incubator at 60°C. The bottles were then taken out after 3, 6, 9 and 12 days respectively. The 1000mg/ml tetracycline stock solution used in step 2.9 was further used to prepare cow milk containing tetracycline in the concentrations 0, 15, 30 and 45ppb. 200µl of the different concentrations, each in duplicates, were added into microwells followed by careful mixing by pipetting up and down. After three minutes the test strips were inserted into the microwells and left there for another three minutes before being taken out and in order to stop the continuation of the process the sample pads were removed. Pictures were taken to document the resulting color intensity of the test- and control lines in order to see whether the assay was still reliable. Every 1 day at 60°C with remained stability of the assay corresponds to 1 month of shelf life under normal storage conditions (room temperature) according to previous studies (Gong et al. 2012).

3. Results

3.1 Characterization of colloidal gold and antibody-gold conjugation

The produced gold nanoparticles were examined using a transmission electron microscope (see figure 5) and it can be seen that the particles were relatively well-dispersed and of slightly varying size and shape, with a mean diameter of around 25-30nm.

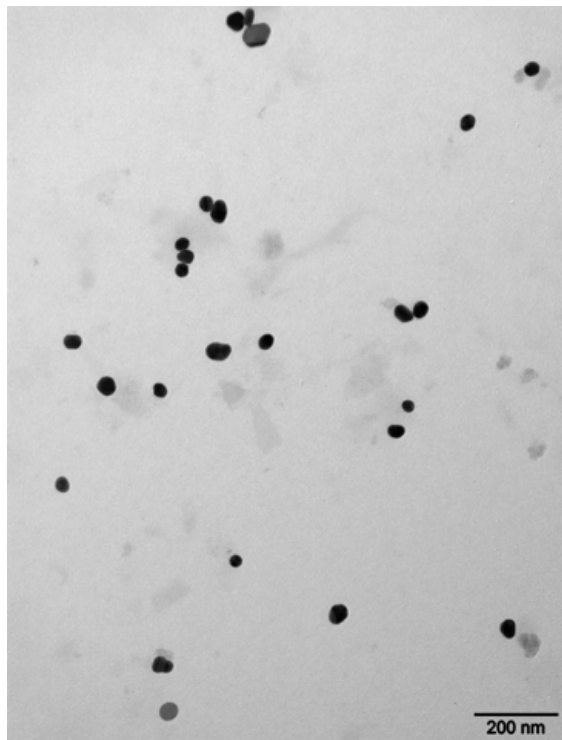


Figure 5. Showing a fraction of the gold nanoparticles present in the colloidal gold solution as viewed with a transmission electron microscope.

An optical density spectrum of the colloidal gold between wavelengths 400nm and 800nm was obtained using a spectrophotometer, as can be seen in figure 6. The peak was found at 530nm.

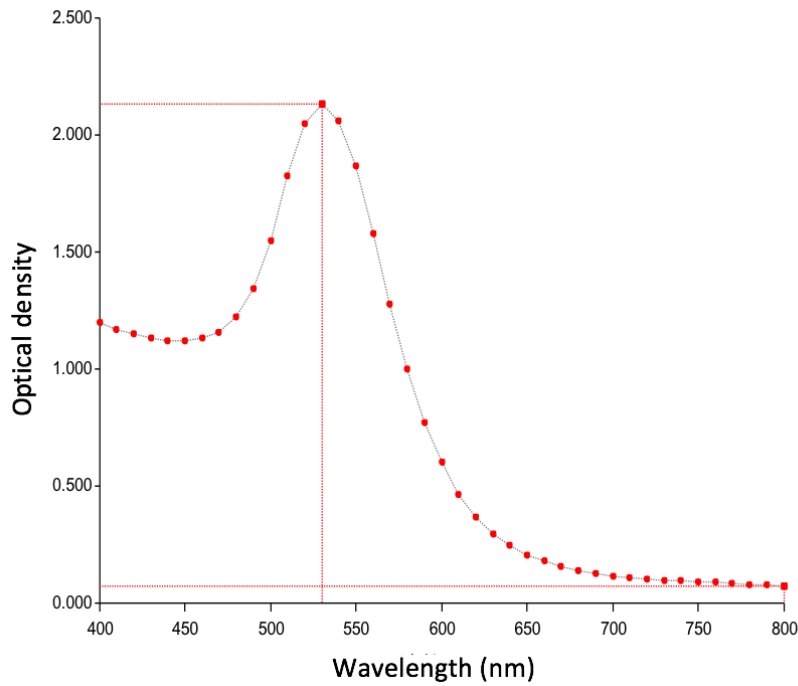


Figure 6. Showing the optical density at different wavelengths for the colloidal gold. The optical density was found to peak at 530nm.

This was used for the determination of the optimal amount of added K_2CO_3 for antibody-gold conjugation, where the amount that generated the highest optical density at a wavelength of 530nm was found to be $8\mu l$ (figure 7).

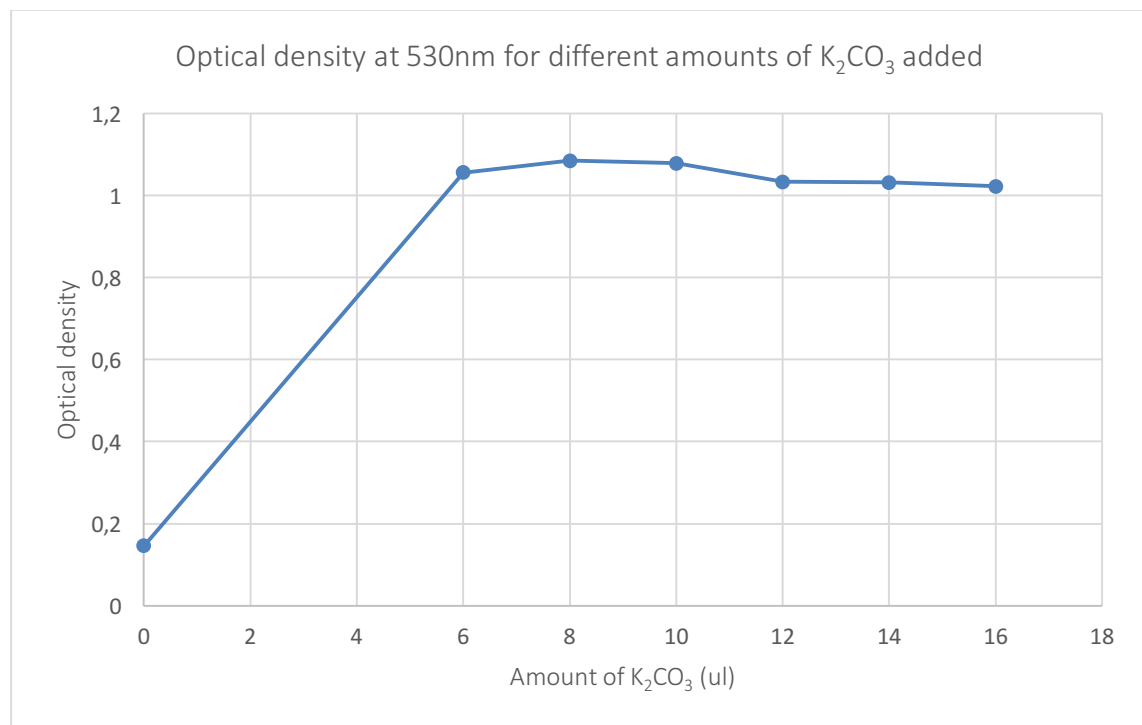


Figure 7. Showing the optical density of the antibody-gold conjugate solution at 530nm for different amounts of added K_2CO_3 . The highest optical density was obtained when $8\mu l$ of K_2CO_3 was added.

3.2 Optimal antigen concentration

Figure 8 shows the resulting color intensities of test lines containing different concentrations of the antigen. The intensity reached a peak at $2\mu\text{g}/\mu\text{l}$, which was also chosen as the optimal concentration since higher color intensities of the test lines for negative samples increases the sensitivity of the assay.

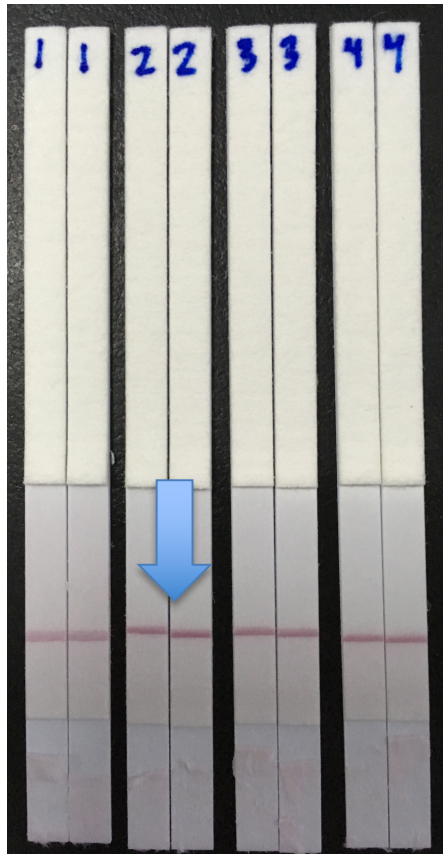


Figure 8. Showing the resulting test line intensity after altering the concentration of the antigen (TC-BSA). In duplicates from the left: 1, 2, 3 and $4\mu\text{g}/\mu\text{l}$. The highest color intensity was obtained at a concentration of $2\mu\text{g}/\mu\text{l}$ (see blue arrow), which was determined to be the optimal one. $5\mu\text{l}$ of detection antibody with concentration $0.08\mu\text{g}/\mu\text{l}$ was used.

3.3 Optimal detection antibody concentration

Figure 9 shows the resulting color intensities of the test lines after addition of 5 μ l of different detection antibody concentrations. The intensity seemed to increase with increasing concentration, but with a barely detectable difference between the two highest concentrations, 0.12 and 0.15 μ g/ μ l. Therefore **0.12 μ g/ μ l** was chosen as the optimal one, as less usage of antibody is preferable from an economical aspect.

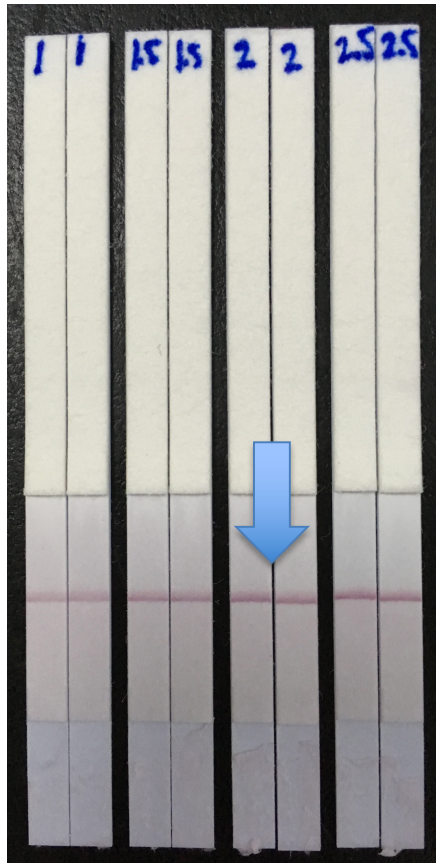


Figure 9. Showing the resulting test line intensity after addition of 5 μ l of detection antibody of different concentrations. From the left in duplicates: 0.06, 0.09, 0.12 and 0.15 μ g/ μ l. The optimal concentration was chosen to be 0.12 μ g/ μ l (see blue arrow). Antigen concentration in test line was 2 μ g/ μ l.

3.4 Optimal detection antibody volume

Figure 10 shows the resulting color intensities of test lines after addition of different volumes of detection antibody with the concentration $0.12\mu\text{g}/\mu\text{l}$. The intensity seemed to increase with an increased volume of added detection antibody, but increased volume also resulted in red staining of the test strip background. To maintain a clean look for the test strip the volume $5\mu\text{l}$ was determined optimal.



Figure 10. Showing the resulting test line intensity after addition of different volumes of detection antibody with the concentration $0.12\mu\text{g}/\mu\text{l}$. From the left in duplicates: 5, 10, 15 and 20 μl . The optimal volume was determined to be $5\mu\text{l}$ (see blue arrow). Antigen concentration in test line was $2\mu\text{g}/\mu\text{l}$.

3.5 Optimal pH in microwell

Figure 11 shows the resulting color intensities of test lines after addition of 5 μ l of Tris-HCl buffers with different pH values. Higher pH values decreased the intensity of the test line. 5 μ l of Tris-HCl buffer with **pH 7.5** was chosen to be the optimal alternative.

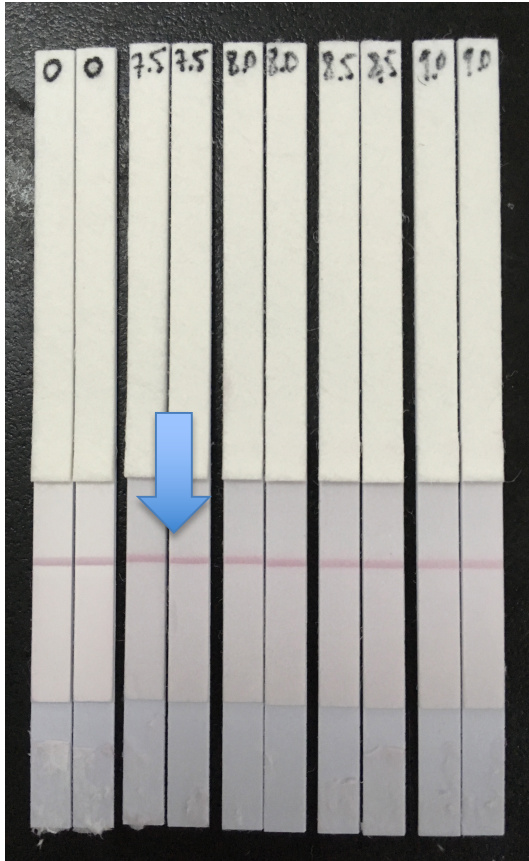


Figure 11. Showing the resulting test line intensities after addition of 5 μ l of Tris-HCl buffers with different pH values. From the left in duplicates: Controls, pH7.5, pH8.0, pH8.5 and pH9.0. The highest color intensity was obtained at pH7.5 (see blue arrow), which was determined to be the optimal one. 5 μ l of detection antibody with concentration 0.12 μ g/ μ l was used and antigen concentration in test line was 2 μ g/ μ l.

3.6 Optimal surfactant in microwell

Figure 12 shows the resulting color intensities of test lines after addition of different volumes of the nonionic surfactant 10% Tween 20. Addition of Tween 20 had a negative effect on the color intensity and was so deemed unsuitable for usage in the assay.

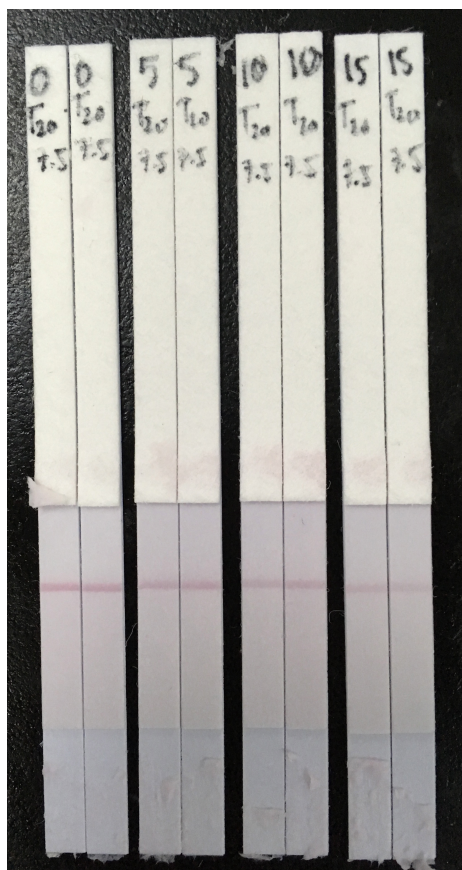


Figure 12. Showing the resulting test line intensities after the addition of different volumes of 10% Tween 20. From the left in duplicates: Controls, 5, 10 and 15µl. 5µl of Tris-HCl pH 7.5, 5µl of detection antibody with concentration 0.12µg/µl and antigen concentration in test line of 2µg/µl was used.

Figure 13 shows the resulting change in milk texture after addition of 5µl of cationic surfactant 5% BAC. After removal of the milk from the microwells there was a clear difference between the control and the one containing BAC. Milk containing BAC formed lumps and stuck to the walls of the microwell. BAC was thus determined not to be suitable for usage in the assay.

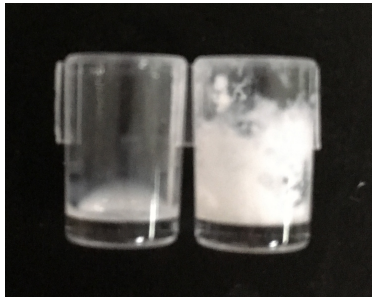


Figure 13. Showing the change in milk texture after the addition of 5 μ l of BAC. After removal of the milk it was seen that the one containing only milk (left microwell) had clean walls while the one also containing BAC (right microwell) had milk lumps stuck to the walls.

Figure 14 shows the resulting color intensities of test lines after addition of different volumes of the amphoteric surfactant 5% Tetronic 1307. There was no visible difference in intensity between the controls and the samples containing 5 μ l of Tetronic 1307. The samples containing 10 and 15 μ l respectively resulted in slightly decreased color intensities. Using **5 μ l of Tetronic 1307** was determined to be the optimal option as it did not have a negative effect on the color intensity and the presence of surfactant in the microwell has been documented to have positive effects on lateral flow immunoassays.

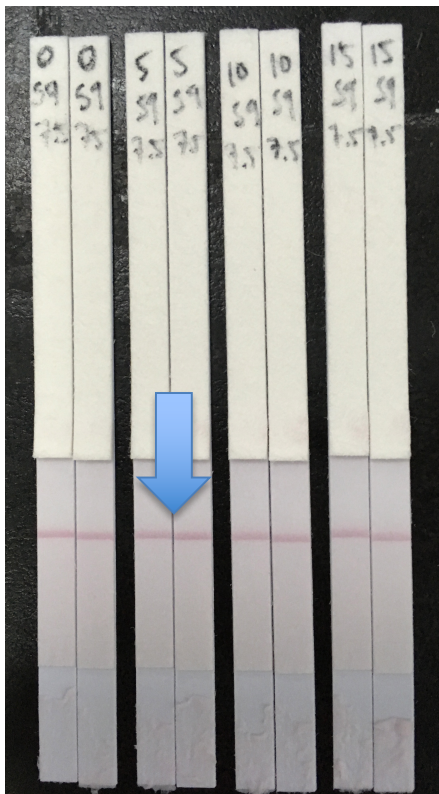


Figure 14. Showing the resulting test line intensities after the addition of different volumes of 5% Tetronic 1307. From the left in duplicates: 0, 5, 10 and 15 μ l. 5 μ l of 5% Tetronic 1307 was determined to be the optimal volume (see blue arrow). 5 μ l of Tris-HCl pH 7.5, 5 μ l of detection antibody with concentration 0.12 μ g/ μ l and antigen concentration in test line of 2 μ g/ μ l was used.

3.7 Test strip detection limit

The test strips and the optimized microwell conditions from previous steps were used on cow milk containing concentrations of tetracycline between 0 and 120ppb in order to find a suitable detection limit of the developed assay. It was intended to find the concentration where the resulting color intensity of the test line was somewhere in between a clearly positive or negative result, meaning a detectable line but not with the same color intensity produced by a completely negative sample. In figure 15 it can be seen that at a concentration of 30ppb visible but weak lines appeared, and so **30ppb** was chosen to be the detection limit.

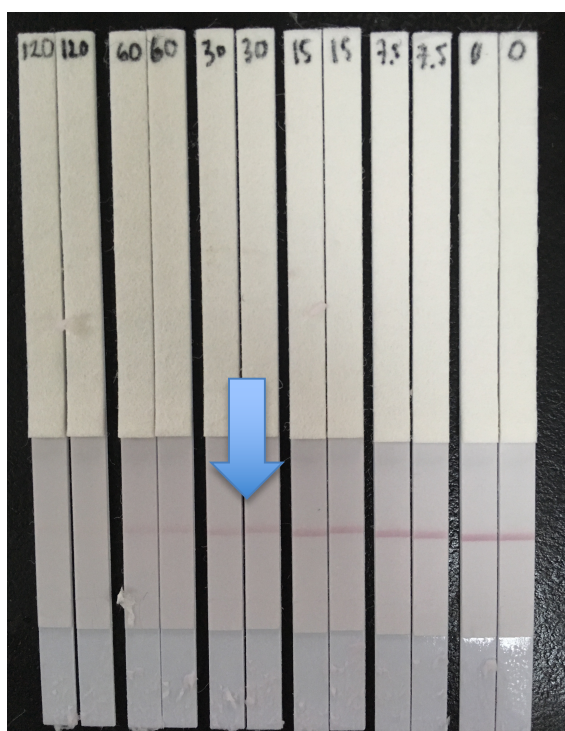


Figure 15. Showing the resulting test line intensities after testing cow milk containing different concentrations of tetracycline. From the left in duplicates: 120, 60, 30, 15, 7.5 and 0ppb. At 30ppb a visible but weak line appeared and that concentration was chosen as the detection limit (see blue arrow). 5 μ l of 5% Tetrac 1307, 5 μ l of Tris-HCl pH 7.5, 5 μ l of detection antibody with concentration 0.12 μ g/ μ l and antigen concentration in test line of 2 μ g/ μ l was used.

3.8 Control line

The aim of the formation of the control line was to find a concentration of the secondary antibody that would result in an equal color intensity as the test line in a sample with a tetracycline concentration of 30ppb (the detection limit). Like this comparison of the intensity of the test line relative to the control line would enable concluding whether the sample contains more or less than 30ppb of tetracycline.

Figure 16 shows the resulting test- and control line intensities at concentrations of tetracycline ranging from 0 to 45ppb when a secondary antibody concentration of 0.25 $\mu\text{g}/\mu\text{l}$ was used. It can be seen that from the sample containing 30ppb tetracycline the intensity of the control line was higher than that of the test line. This indicates that the concentration of the secondary antibody is too high and thus not suitable for test strips with a desired detection limit of 30ppb.

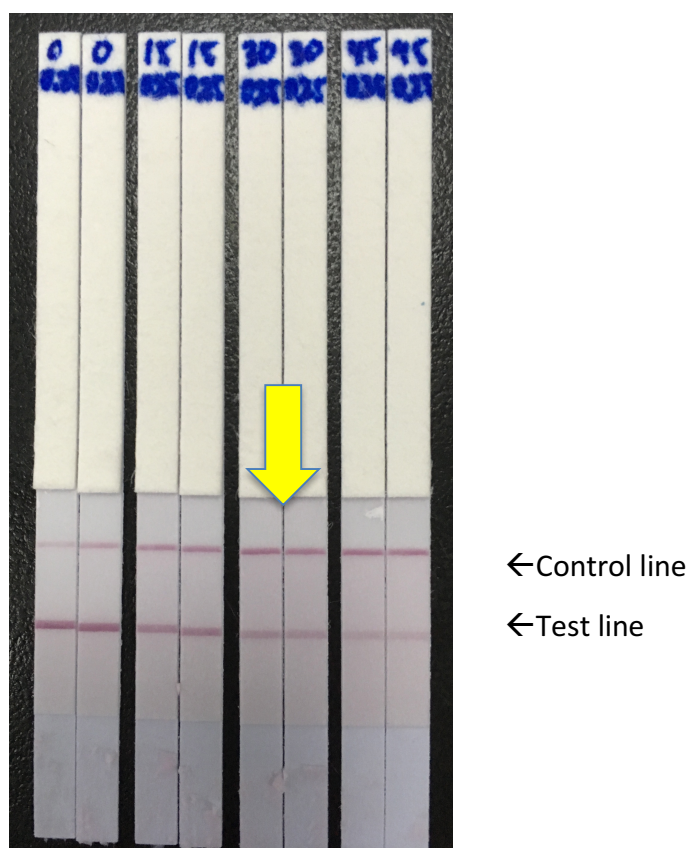


Figure 16. Showing the resulting test- and control line intensities in samples with different concentrations of tetracycline when a secondary antibody concentration of 0.25 $\mu\text{g}/\mu\text{l}$ was used. From the left in duplicates: 0, 15, 30 and 45ppb. At 30ppb the intensity of the control line was higher than that of the test line (see yellow arrow). 5 μl of 5% Tetronic 1307, 5 μl of Tris-HCl pH 7.5, 5 μl of detection antibody with concentration 0.12 $\mu\text{g}/\mu\text{l}$ and antigen concentration in test line of 2 $\mu\text{g}/\mu\text{l}$ was used.

Figure 17 shows the resulting test- and control line intensities at concentrations of tetracycline ranging from 0 to 45ppb when a secondary antibody concentration of 0.15 $\mu\text{g}/\mu\text{l}$ was used. It can be seen that from the sample containing 30ppb tetracycline the intensity of the control line was higher than that of the test line. This indicates that the concentration of the secondary antibody is still too high and thus not suitable for test strips with a desired detection limit of 30ppb.

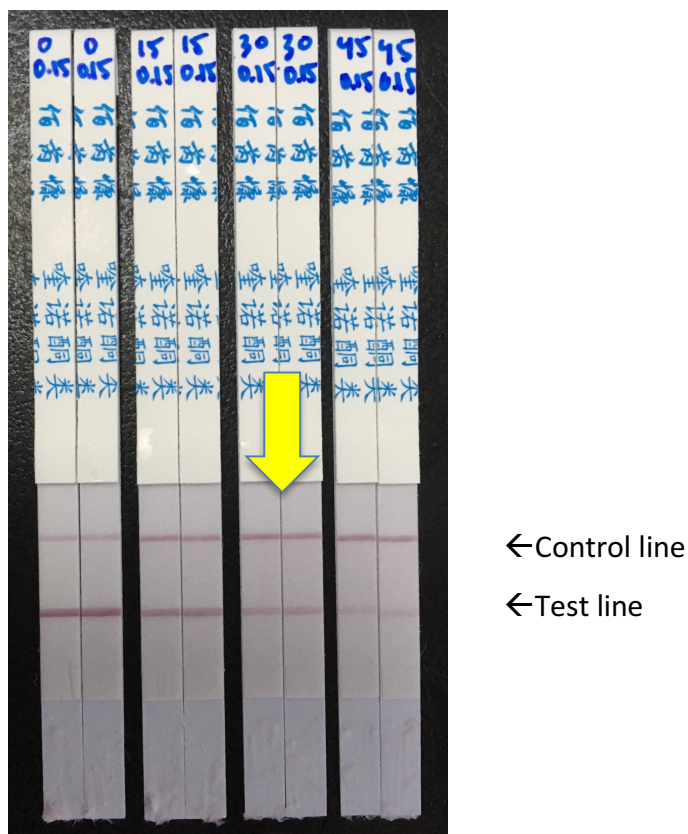
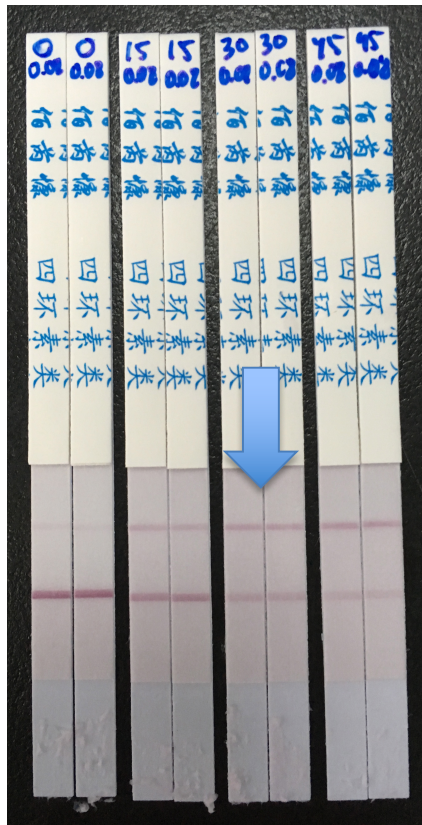


Figure 17. Showing the resulting test- and control line intensities in samples with different concentrations of tetracycline when a secondary antibody concentration of $0.15\mu\text{g}/\mu\text{l}$ was used. From the left in duplicates: 0, 15, 30 and 45ppb. At 30ppb the intensity of the control line was higher than that of the test line (see yellow arrow). $5\mu\text{l}$ of 5% Tetronic 1307, $5\mu\text{l}$ of Tris-HCl pH 7.5, $5\mu\text{l}$ of detection antibody with concentration $0.12\mu\text{g}/\mu\text{l}$ and antigen concentration in test line of $2\mu\text{g}/\mu\text{l}$ was used.

Figure 18 shows the resulting test- and control line intensities at concentrations of tetracycline ranging from 0 to 45ppb when a secondary antibody concentration of $0.08\mu\text{g}/\mu\text{l}$ was used. It can be seen that from the sample containing 30ppb tetracycline the intensity of the control line was highly similar to that of the test line. This indicates that a secondary antibody concentration of **$0.08\mu\text{g}/\mu\text{l}$** is suitable for test strips with a desired detection limit of 30ppb.



←Control line

←Test line

Figure 18. Showing the resulting test- and control line intensities in samples with different concentrations of tetracycline when a secondary antibody concentration of $0.08\mu\text{g}/\mu\text{l}$ was used. From the left in duplicates: 0, 15, 30 and 45ppb. At 30ppb the intensity of the control line was equal to that of the test line (see blue arrow). $5\mu\text{l}$ of 5% Tetronic 1307, $5\mu\text{l}$ of Tris-HCl pH 7.5, $5\mu\text{l}$ of detection antibody with concentration $0.12\mu\text{g}/\mu\text{l}$ and antigen concentration in test line of $2\mu\text{g}/\mu\text{l}$ was used.

Figure 19 shows an adapted version of the Shenzhen Bioeasy Biotechnologies Co. Instruction manual for the use of their melamine test strip. The principle is exactly the same for the developed tetracycline test strip. If no color is visible on the control line the assay is considered invalid. If the test line has a higher color intensity than the control line the sample is considered negative ($<30\text{ppb}$ tetracycline). If the control line and the test line has the same color intensity the sample would contain approximately 30ppb tetracycline. If the test line has a weaker color intensity than the control line the sample is considered positive ($>30\text{ppb}$ tetracycline). Comparing figure 18 with this manual shows that the developed assay complies with these guidelines.

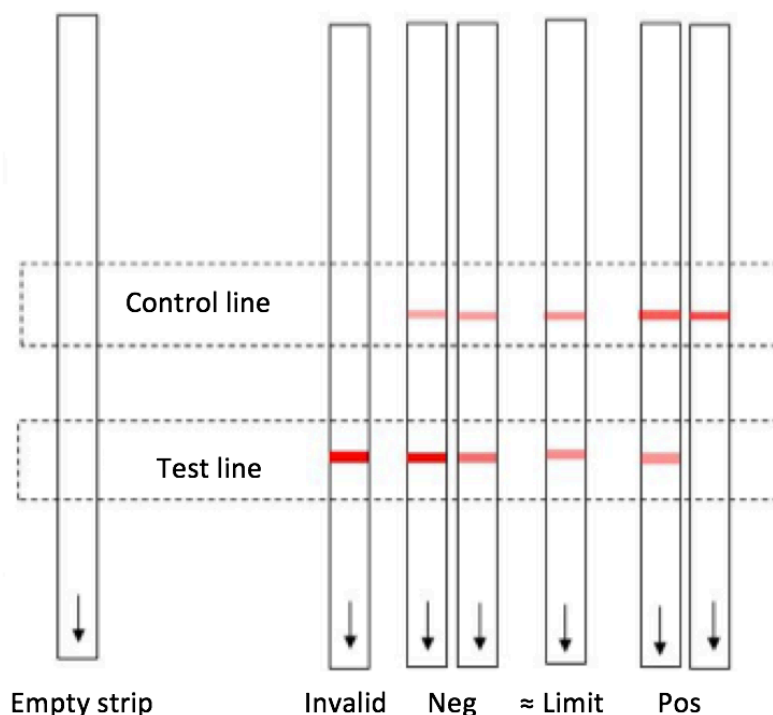


Figure 19. Showing the instructions for interpretation of the test strips as adapted from the Shenzhen Bioeasy Biotechnologies Co. Manual for use of their melamine test strip. If no color is visible on the control line the assay is considered invalid. If the test line has a higher color intensity than the control line the sample is considered negative (under the detection limit). If the control line and the test line has the same color intensity the sample would contain a concentration of the analyte around the detection limit of the assay. If the test line has a weaker color intensity than the control line the sample is considered positive (over the detection limit).

3.9 Finished product

Figure 20 shows the appearance of the finished product. Desiccant bottles were filled with sets of eight microwells and test strips used together for detection of tetracycline in cow milk. Users of the product only need to add 200 μ l of milk samples into the microwell and mix carefully with a pipette followed by waiting three minutes before inserting a test strip and then another three minutes before taking the strip out. Comparisons with an instruction manual such as the one showed in figure 19 then allows for determination of whether the sample is positive or negative.



Figure 20. Showing the finished product and its distribution. Each plastic desiccant bottle (left) containing a set of eight covered microwells with freeze-dried components and eight test strips (right).

3.10 Accelerated stability testing

Figure 21 shows the resulting test- and control line intensities for the batch of test strips and microwells that had been stored at 60°C for 12 days. For the samples containing 0 and 15ppm (negative) the test lines had a higher color intensity than the control lines, at the detection limit (30 ppb) the intensities of the test- and control line was similar and at 45ppb (positive) the intensity of the test line was weaker than that of the control line, all in accordance with the desired principal of the assay (see figure 19). Conversion of stability for 12 days at 60° into normal storage conditions indicates that the developed assay should have an expected shelf life of at least 12 months.

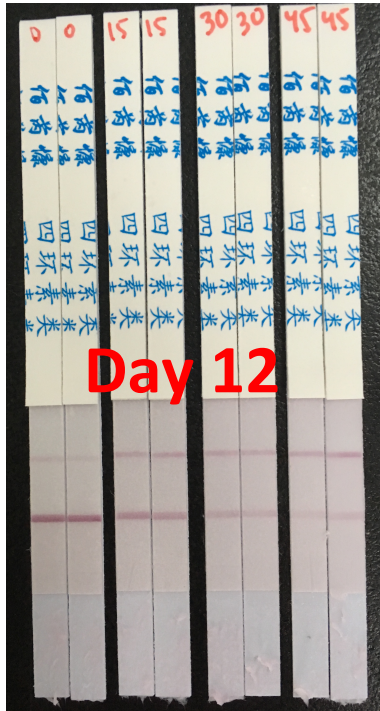


Figure 21. Showing the results from the 12th and final day of the accelerated stability test. The test- and control line intensities in samples with different concentrations of tetracycline can be seen. From the left in duplicates: 0, 15, 30 and 45ppb.

4. Discussion

The aim of this project was to develop and optimize a commercially utilizable micro-well based lateral flow immunoassay for the detection of tetracycline in cow milk. The optimized conditions leading to the final product are summarized as follows: Each test strip included a test line with an **antigen (TC-BSA) concentration of 2 $\mu\text{g}/\mu\text{l}$** and a control line with a **secondary antibody (goat anti-mouse IgG) concentration of 0.08 $\mu\text{g}/\mu\text{l}$** . Each microwell contained **5 μl of detection antibody (anti-TC) with a concentration of 0.12 $\mu\text{g}/\mu\text{l}$** as well as **5 μl of Tris-HCl buffer with pH 7.5** and **5 μl of the surfactant Tetronic 1307**. These conditions resulted in an assay **detection limit of 30ppb** tetracycline and an expected **shelf life of at least 12 months**. The assay time is around **6-7 minutes**.

The characteristics of the developed assay are of comparable standards with other lateral flow immunoassays. For example, the fully commercialized Charm ROSA Tet test is developed by Charm Sciences, Inc. and detects tetracycline in milk. The assay time is 8 minutes and the detection limit is 100ppb for tetracycline (Charm Sciences 2017). Comparing with the developed assay the assay times are very similar but there is a significant difference in the sensitivity. The Charm ROSA Tet test is suitable for the European market since the MRL requirements for tetracycline residues is 100ppb. The developed assay could also be used in the European market but might be considered to be unnecessarily sensitive, leading to milk being labeled as positive for tetracycline and so rejected despite containing less tetracycline than the actual MRL. The Charm ROSA Tet test is on the other hand too insensitive for the Chinese market where the MRL is 50ppb. A detection limit of 30ppb, which is the case for the developed assay, works well for the Chinese market since it can be considered to have a reasonable safety margin.

The developed assay does not require the use and preparation of a conjugate pad, which significantly reduces the complexity of test strip assembling. Using a conjugate pad also implies the need for the sample to flow through more layers, increasing the risk for loss of detection antibody or clogging of samples during transportation through the strip, affecting the sensitivity. The layer on layer strip that the conjugate pad also causes entails the usage of a plastic housing to keep everything in place, which increases the material costs (Kabri et al. 2010). The developed assay instead uses a microwell containing the detection antibody, making the preparation of the assay more straight-forward, while not compromising the sensitivity.

Since the colloidal gold is directly correlated with the sensitivity of the assay the production step is of very high importance. In figure 5 the resulting gold nanoparticles can be seen and the sizes and shapes of the individual particles differ

to a certain extent from each other. In this aspect there is room for improvement for the assay since gold nanoparticles of different sizes move through the test strips at different speeds, resulting in differing sensitivities from assay to assay. (Innova Biosciences 2017).

During the development process three different kinds of surfactants were added to the microwells to investigate which had the best effect on the assay. Addition of the cationic surfactant benzalkonium chloride seemed to cause lump formation in the milk sample (see figure 13). Addition of non-ionic surfactant Tween 20 and amphoteric surfactant Triton 1307, both non-ionic surfactants, did not have that effect on the milk. Milk proteins such as casein are normally negatively charged and are present as colloidal solutions (Tetra Pak 2017). It is possible that by addition of a cationic surfactant the colloidal solution is destabilized and starts to coagulate because the proteins lose their charge and stop repelling each other, causing the milk lumps. It is therefore concluded that cationic surfactants are not suitable for this kind of milk-directed assay.

The developed assay lacks some necessary steps in order to be fully acknowledged commercially. The test strip is stated to have a detection limit at 30ppb tetracycline but in order to verify its accuracy the results from the assay for several different kinds of milk samples should be compared with those from a more sophisticated method such as liquid chromatography-mass spectrometry (LC-MS) that can provide exact concentration figures (Han et al. 2016). This step is of great importance to confirm the reliability of the test strip.

Another aspect that needs to be considered is that of cross-reactivity. It is not known if structures closely related to tetracycline might interfere or be detected in the assay. This weakness could be prevented by testing for cross-reactivity, in this case by adding the known tetracycline analogues chlortetracycline and oxytetracycline to milk samples and seeing whether cross-reactivity occurs. If that should be the case the assay cannot be used for tetracycline specifically but could instead be used as a screening test for antibiotics of the tetracycline family in general. Should a sample show positive results it could be sent to a lab for further testing using LC-MS which would provide the exact identity and concentration of the contaminant.

4.1 Future work

In cases where a farmer or veterinary wants to check residues in milk from a specific cow known to have been given a certain kind of antibiotic the kind of assay developed works well. There are however situations where the milk is to be

screened for a lot of different kinds of antibiotics, for example in milk analyzing laboratories, where no previous knowledge about what the cattle has been given is at hand. In those cases it would be more efficient to use test strips capable of screening for several antibiotics simultaneously. A market would therefore be available for multiplex assays, in other words test strips with several different test lines. This requires assays of a different approach since the different antibiotics would most likely have different detection limits.

The developed test strip is read off visually and is therefore appropriate for qualitative measurements, requiring positive samples to be sent to laboratories for more extensive testing in order to obtain more exact results. More effort could be put into making it a more quantitative assay by employing colorimetric measurements that convert test line intensities into approximate concentrations.

Since the lateral flow immunoassays have a commercial purpose the economical aspect is of constant consideration. More work could be put into cutting the production costs and one of the major expenses is antibodies used in the assay. Instead of using passive adsorption methods to create the antibody-gold conjugates slightly more complex covalent conjugation should be further looked into since it uses significantly less of the antibody (Innova Biosciences 2017).

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