CÁSSIO MENDES FONTES

QUANTIFICAÇÃO POR IMAGEM DE TESTES IMUNOCROMATOGRÁFICOS DE FLUXO LATERAL DESENVOLVIDOS PARA A DETECÇÃO DE HIV, HCV E FOB
QUANTIFICAÇÃO POR IMAGEM DE TESTES IMUNOCROMATOGRAFÍCOS DE FLUXO LATERAL DESENVOLVIDOS PARA A DETECÇÃO DE HIV, HCV E FOB

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Cássio Mendes Fontes: Quantificação Por Imagem de Testes Imunocromatográficos de Fluxo Lateral Desenvolvidos Para A Detecção de HIV, HCV e FOB, Masters Dissertation, © October 2013
In the middle of difficulty lies opportunity

(Albert Einstein).

Dedicado em memória de Dindinha

(In loving memory of Dindinha).
ABSTRACT

Lateral Flow immunoassays, have been developed in the late seventies, early eighties and due to its characteristics such as low cost, unnecessary use of costly readers for obtaining a qualitative result, easy to use and to interpret formats, they became very popular worldwide. Due to their unique characteristics, they allowed health organization to bring point-of-care testing to populations that have rarely seen medical care. Although several advantages may be pointed out, there are two main set backs on the technology; the lack of quantification possibilities for the test devices and possible human error, which may occur in weak signal results. Since most of the tests use colored conjugates such as colloidal gold, reading systems for such tests depend on image based recognition for final result quantification or assured qualitative results. In this work we not only developed three lateral flow tests, for the detection of HIV, HCV and occult blood in feces (FOB), but we analyzed images from final tests, in order to access the possibility of quantifying the test’s final result and encountered a linear relation between the final result and the amount of analyte present in the sample.

RESUMO

Ensaios imunocromatográficos de fluxo lateral foram desenvolvidos ao final dos anos setenta, início dos anos oitenta. Devido a suas características como baixo custo, dispensam a necessidade de leituras com custo elevado para se obter resultados qualitativos, formato de fácil uso e interpretação, eles se tornaram extremamente populares mundialmente. Devido suas características únicas, eles possibilitaram que organizações de saúde levasssem ensaios point-of-care a populações que dificilmente teriam acesso a cuidados de saúde. Apesar de várias vantagens serem enumeradas, existem duas grandes fraquezas na plataforma em si. A primeira é a falta de métodos de quantificação para os ensaios e a segunda é a possibilidade de erro humano durante a interpretação dos resultados especialmente os que apresentam sinal fraco. Como a maioria dos testes fazem uso de conjugados a base ouro coloidal, sistemas de leitura dependem de reconhecimento de imagem para a quantificação final ou para a obtenção de resultados qualitativos seguros. Nesse trabalho alem de terem sido desenvolvidos três ensaios imunocromatográficos de fluxo lateral para a detecção de HIV, HCV e sangue oculto em fezes (FOB), também foram analisadas as imagens dos resultados dos testes, de forma a estudar a possibilidade de quantificação dos ensaios. Uma relação linear entre o resultado final
da quantificação do teste e a quantidade de analito na amostra foi encontrada.
To everything there is a season, and a time to every purpose under the heaven: A time to be born, and a time to die; a time to plant, and a time to pluck up that which is planted...(Ecclesiastes 3:1-2)

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Muito obrigado a todos.
RESUMO ESTENDIDO

Esse capítulo faz um resumo geral de todos os temas abordados no trabalho bem como dos resultados na, língua portuguesa. Os capítulos seguintes detalham os dados, metodologias, e resultados obtidos.

1. CARACTERÍSTICAS GERAIS

A plataforma de testes, utilizada nos ensaios imunocromatográficos de fluxo lateral, também conhecidos como testes rápidos, é o resultado da convergência de diversas tecnologias que vem se aprimorando desde a década de 50 [36]. Apesar de bem estabelecida, e apresentar elevados níveis de confiança, apenas em anos recentes, viu-se um crescimento exponencial do uso dessa tecnologia aplicada ao diagnóstico de diversas condições clínicas e patológicas [21]. Existem grandes vantagens no uso dessa plataforma de testes como:

• Tecnologia madura e bem estabelecida;
• Dispositivos de fácil uso;
• Fabricação simples, pois os processos e equipamentos já encontram-se desenvolvidos e padronizados;
• Pode viabilizar testes com elevada sensibilidade e especificidade.

Uma das maiores desvantagens da tecnologia é a falta de metodologias para quantificar os resultados finais dos ensaios [26]. Esse trabalho vem exatamente explorar essa lacuna. Ele explora o comportamento de testes-rápidos ao serem quantificados comparando com amostras de concentrações conhecidas podendo resultar em metodologias de quantificação ou fornecer ao menos o título das amostras.

2. COMPOSIÇÃO DO TESTE

Os dispositivos teste são compostos por membranas superpostas, partículas detectores e material biológico. Cada membrana tem uma determinada função e característica [13]. A figura 2.1 mostra todos os componentes de um teste-rápido tradicional. Esses componentes são:

• Material de suporte;
• Almofada do conjugado;
• Almofada da amostra;
• Matriz reacional;
• Filtro absorvente;
• Material biológico para as linhas teste e controle;
• Partículas detectoras.

3. Formatos de Teste

Testes-rápidos podem ser separados em dois grandes grupos de testes. Os testes que detectam antígenos e os testes que detectam anticorpos nas amostras [36].

DETEÇÃO DE ANTÍGENOS: Testes que realizam a deteção de antígenos fazem uso de um par de anticorpos específicos para capturar a partícula a ser detectada. Um dos anticorpos é imobilizado na matriz reacional e o seu par é conjugado a partícula de detecção. Ao ser adicionada, a amostra reconstitui a partícula de detecção imobilizada na almofada do conjugado e reage com ela simultaneamente. Se presente, o antígeno é capturado pelo anticorpo ligado a partícula. O complexo partícula, anticorpo, antígeno migra pela matriz reacional até alcançar a zona teste. Na zona teste, o segundo anticorpo imobilizado na matriz reacional captura o complexo migrante, originando um sinal visual ou interpretado por leituras. O excesso de conjugado é capturado pelas proteínas imobilizadas na região controle.

DETECÇÃO DE ANTICORPOS: Testes que realizam a deteção de anticorpos, fazem uso de um antígeno específico imobilizado a matriz reacional. Proteínas conjugadas a partícula detectora que se ligam ao anticorpo de interesse na amostra também são utilizadas. Ao ser adicionada, a amostra reconstitui o conjugado. Se presentes, os anticorpos específicos se ligam ao conjugado. Esse complexo anticorpo conjugado migra pela matriz reacional e se liga ao antígeno imobilizado na região da zona teste. O excesso de conjugado é capturado pelas proteínas imobilizadas na região controle.

4. HIV

4.1. Relevancia de seu Diagnóstico

Em dezembro de 2003 existiam aproximadamente 40 milhões de pessoas vivendo com o HIV no mundo. O diagnóstico da doença tem melhorado imensamente o cuidado com os pacientes e consequentemente sua qualidade de vida.
O diagnóstico baseia-se na deteção de antígenos específicos ou de anticorpos contra outros antígenos do HIV. Vários ensaios detectam anticorpos específicos contra o HIV, entre esses testes estão os testes rápidos e especificamente o testes desenvolvido nesse trabalho [12].

4.2. O Dispositivo Teste
O teste rápido para a detecção do HIV desenvolvido nesse trabalho, faz uso da técnica de ensaio sequencial. Antígenos do HIV são imobilizados na matriz reacional. O ouro coloidal é conjugado a proteína A para realizar a revelação dos anticorpos que foram capturados na linha teste. O excesso de conjugado continua migrando pela membrana e é capturado pela linha controle indicando o correto funcionamento do teste.
Diversos ajustes e padronizações foram realizados durante o desenvolvimento do teste. Detalhes do desenvolvimento do dispositivo podem ser vistos no capítulo 3.

4.3. A Quantificação dos Resultados
O estudo da quantificação dos resultados dos testes rápidos foi realizado no capítulo 4. Foram utilizadas amostras sintéticas e de pacientes reais para se construir uma curva de calibração e obter concentrações valores de título de anticorpos a partir dessa curva de calibração. Os resultados do método mostraram boa concordância com os obtidos através de outra metodologia.

5. HCV

5.1. Relevancia de seu Diagnóstico
A hepatite C é uma doença que afeta 200 milhões de pessoas mundialmente. Ela é uma doença de elevado risco pois ela apresenta elevado risco de complicações grave aos infectados alem de apresentar elevada prevalência [27]. Casos crônicos da doença podem levar a cirrose, carcinoma hepatocelular e doenças terminais do fígado. O patógeno apresenta elevado índice de mutação, o que permite ao vírus escapar a resposta imune do organismo [7].

5.2. O Dispositivo Teste
O teste rápido para a detecção do HCV desenvolvido nesse trabalho, faz uso da técnica de ensaio sequencial, da mesma forma que o HIV. Antígenos do HCV são imobilizados na matriz reacional. O ouro coloidal é conjugado a proteína A para realizar a revelação dos anticorpos que foram capturados na linha teste. O excesso de conjugado continua migrando pela membrana e é capturado pela linha controle indicando o correto funcionamento do teste.
Diversos ajustes e padronizações foram realizados durante o desenvolvimento do teste. Detalhes do desenvolvimento do dispositivo podem ser visto no capítulo 3.

5.3. A Quantificação dos Resultados

O estudo da quantificação dos resultados dos testes rápidos foi realizado no capítulo 4. Da mesma forma que o HIV, foram utilizadas amostras sintéticas e de pacientes reais para se construir uma curva de calibração e obter concetrações valores de título de anticorpos a partir dessa curva de calibração. Os resultados do método mostraram boa concordância com os obtidos através de outra metodologia.

6. FOB

6.1. Relevância de seu Diagnóstico

A detecção do câncer colorectal em paciente assintomáticos é a melhor forma de se melhorar o prognóstico da doença. A pesquisa de sangue oculto na fezes é hoje o método de triagem anterior a aplicação da colonoscopia, que é o padrão ouro na pesquisa de câncer colorectal [32]. Apesar de ser o padrão ouro, a colonoscopia apresenta diversos riscos aos pacientes como perfuração do intestino sem considerar o desconforto do paciente durante a realização do procedimento, restringindo seu uso como método de triagem [31].

6.2. O Dispositivo Teste

O teste rápido para a detecção de hemoglobina em fezes desenvolvido nesse trabalho, faz uso da técnica de sanduíche, diferentemente do HIV e HCV. Anticorpos específicos para a hemoglobina são imobilizados na matriz reacional e seu par complementar é conjugado ao ouro coloidal. Ao ser adicionada, a amostra reconstitui o ouro coloidal e se presente o antígeno é capturado pelo conjugado. O complexo ouro coloidal, anticorpo e antígeno migra pela matriz reacional sendo capturado pelo anticorpo imobilizado na região teste. O excesso de conjugado é capturado na região controle por um anticorpo espécie específico.

Diversos ajustes e padronizações foram realizados durante o desenvolvimento do teste. Detalhes do desenvolvimento do dispositivo podem ser visto no capítulo 3.

6.3. A Quantificação dos Resultados

O estudo da quantificação dos resultados do FOB foi realizado no capítulo 4. Foram utilizados controles de concentração conhecida para se construir uma curva de calibração. Infelizmente os resultados não foram satisfatórios, principalmente devido ao background do ouro coloidal na matriz reacional.

Uma relação linear entre o resultado final da quantificação do teste e a quantidade de analito na amostra foi encontrada.
# CONTENTS

**i INTRODUCTION**

1 INTRODUCTION  

**ii BACKGROUND KNOWLEDGE**

2 PREVIOUS WORKS  
  2.1 A Little Background Information  
  2.2 Lateral Flow Immunoassays  
  2.2.1 Architecture of a Lateral Flow Immunoassay  
  2.2.2 Commonly Used Materials and Processes in Development and Manufacturing of Lateral Flow Immunoassays  
  2.3 Reader Systems in Lateral Flow Assays  
  2.4 HIV diagnostic  
  2.4.1 Structure of HIV  
  2.4.2 How HIV infects target cells  
  2.4.3 Different HIV subtypes  
  2.4.4 Serological profile of HIV infection  
  2.4.5 HIV Diagnostic Tests  
  2.5 Hepatitis C diagnostic  
  2.5.1 Structure of HCV  
  2.5.2 How HCV infects target cells and replicates  
  2.5.3 HCV Diagnostic Tests  
  2.6 Immunological Detection of Haemoglobin in Fecal Matter  
  2.6.1 Guaiac based tests  
  2.6.2 Heme Porphyrin tests  
  2.6.3 Immunochemical tests  
  2.6.4 Interpretation of occult blood tests  
  2.7 Quantification of Lateral Flow Device Tests  
  2.7.1 Image Analysis Techniques for Test Quantification  

**iii DEVELOPED WORK**

3 TEST DEVELOPMENT  
  3.1 Rapid Test Development  
  3.2 Test Development Steps  
  3.3 HIV Detection Test  
  3.3.1 Test Format Selection  
  3.3.2 HIV Antigen Selection  
  3.3.3 Label Production  
  3.3.4 Protein Concentration Adjustment  
  3.4 HCV Detection Test  
  3.4.1 Test Format Selection  
  3.4.2 HCV Antigen Selection
3.4.3 Label Production ........................................... 69
3.4.4 Protein Concentration Adjustment ................. 69
3.5 FOB Detection Test ........................................... 72
  3.5.1 Test Format Selection ................................. 72
  3.5.2 FOB Antibody Selection ............................. 73
  3.5.3 Label Production ......................................... 73
  3.5.4 Protein Concentration Adjustment ................. 74
4 RAPID TEST QUANTIFICATION ............................... 87
  4.1 Challenges Regarding Test Quantification ............ 87
    4.1.1 Positioning Errors .................................... 88
    4.1.2 Off-Axis Versus Confocal Measurements .......... 88
    4.1.3 Accurate, Sufficient and Homogeneous Illumination of the Sample .................. 89
    4.1.4 Numerical Aperture, Field of View and Sensitivity ........... 89
    4.1.5 Calibration Curve ..................................... 89
  4.2 Image Acquisition and Quantification ................. 89
    4.2.1 Quantification Procedure and Results ............... 90
  4.3 Quantifying the HIV Test ................................ 91
    4.3.1 Calibration Curve ..................................... 91
    4.3.2 Method for Quantifying the Test .................. 93
    4.3.3 Quantifying Real Patient Samples ................. 94
  4.4 Quantifying the HCV Test ................................ 95
    4.4.1 Calibration Curve ..................................... 95
    4.4.2 Method for Quantifying the Test .................. 96
    4.4.3 Quantifying Real Patient Samples ................. 96
  4.5 Quantifying the FOB Test ................................ 97
    4.5.1 Calibration Curve ..................................... 97
    4.5.2 Method for Quantifying the Test .................. 97
    4.5.3 Quantifying Real Patient Samples ................. 98
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Rapid Test for the detection of haemoglobin in fecal mater prior, during and after the test is ran</td>
<td>26</td>
</tr>
<tr>
<td>2.1</td>
<td>Lateral Flow Test Components [1]</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Direct Sandwich Assay Test Configuration [22]</td>
<td>34</td>
</tr>
<tr>
<td>2.3</td>
<td>Competitive Assay Test Configuration [22]</td>
<td>35</td>
</tr>
<tr>
<td>2.4</td>
<td>Outline of a lateral flow device production process [22]</td>
<td>36</td>
</tr>
<tr>
<td>2.5</td>
<td>Commercially available nitrocellulose membrane roll [2]</td>
<td>38</td>
</tr>
<tr>
<td>2.6</td>
<td>Commercially available nitrocellulose membrane sheets cut to size [2]</td>
<td>39</td>
</tr>
<tr>
<td>2.7</td>
<td>Colloidal gold molecular structure [35]</td>
<td>40</td>
</tr>
<tr>
<td>2.8</td>
<td>(a - b) Different views of a RDT reader prototype installed on an Android phone (Samsung Galaxy S II). This attachment can be repeatedly attached/detached to the cellphone body without the need for fine alignment and modification. (c - d) Schematic diagrams of the designed optical RDT reader attachment are shown. Depending on the format of the diagnostic test, users can switch between the two illumination schemes (reflection vs. transmission) to acquire an image of the RDT with high contrast. This image is then rapidly processed within less than 0.2 sec/image through a custom-developed application running on the cellphone to generate an automated report that consists of test validation and reading of the diagnostic results as well as quantification of the test line’s color intensities [20].</td>
<td>48</td>
</tr>
<tr>
<td>3.1</td>
<td>Lateral flow test device development steps</td>
<td>54</td>
</tr>
<tr>
<td>3.2</td>
<td>Gold nanoparticles macroscopic appearance after synthesis</td>
<td>60</td>
</tr>
<tr>
<td>3.3</td>
<td>Determination of protein needed to stabilize gold</td>
<td>62</td>
</tr>
<tr>
<td>3.4</td>
<td>Autokun sprayer for test and control line dispensing</td>
<td>68</td>
</tr>
<tr>
<td>3.5</td>
<td>Autokun guillotine cutter for cutting the master sheets into test strips</td>
<td>69</td>
</tr>
<tr>
<td>3.6</td>
<td>ROC curves of HIV batch testing using finalized test devices</td>
<td>70</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>3.7</td>
<td>ROC curves of batch testing using finalized test devices</td>
<td></td>
</tr>
<tr>
<td>3.8</td>
<td>Tray freeze dryer from <em>Liotop</em>®</td>
<td></td>
</tr>
<tr>
<td>3.9</td>
<td>Lateral flow strip with test and control lines</td>
<td></td>
</tr>
<tr>
<td>3.10</td>
<td>ROC curves of batch testing using finalized test devices</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Intensity profile of a test’s acquired image</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>Test’s cropped region isolating the reaction matrix</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>Test image after initial cropping and conversion to gray scale</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>Intensity profile of test’s acquired image after initial cropping and conversion to gray scale</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>Test image after initial cropping and conversion to gray scale</td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>Intensity profile of test’s acquired image after initial cropping and conversion to gray scale</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Intensity profiles of the HIV calibration test battery with samples of known concentration</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>Cropped regions of an HIV test battery with samples presented in table 4.1. The first image corresponds to the first sample in the table and the last image corresponds to the last sample</td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>Calibration curve obtained adding the pixel values of the test zone area for the HIV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the $\log_2$ of the ELISA result with the intensity value obtained from the image</td>
<td></td>
</tr>
<tr>
<td>4.10</td>
<td>Calibration curve obtained averaging the pixel values of the test zone area for the HIV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the $\log_2$ of the ELISA result with the intensity value obtained from the image</td>
<td></td>
</tr>
<tr>
<td>4.11</td>
<td>Intensity against ELISA values obtained with patient sample, plotted together with calibration curve previously determined</td>
<td></td>
</tr>
<tr>
<td>4.12</td>
<td>Intensity profiles of the HCV calibration test battery with samples of known concentration</td>
<td></td>
</tr>
<tr>
<td>4.13</td>
<td>Cropped regions of an HCV test battery with samples presented in table 4.3. The first image corresponds to the first sample in the table and the last image corresponds to the last sample</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.14 Calibration curve obtained adding the pixel values of the test zone area for the HCV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the \( \log_2 \) of the ELISA result with the intensity value obtained from the image.

Figure 4.15 Calibration curve obtained averaging the pixel values of the test zone area for the HCV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the \( \log_2 \) of the ELISA result with the intensity value obtained from the image.

Figure 4.16 Intensity against ELISA values obtained with patient sample, plotted together with calibration curve previously determined.

Figure 4.17 Cropped regions of an FOB test battery with samples presented in table 4.5. The first image corresponds to the first sample in the table and the last image corresponds to the last sample.

Figure 4.18 Intensity profiles of the FOB calibration test battery with samples of known concentration.

Figure 4.19 Calibration curve obtained adding the pixel values of the test zone area for the FOB tests. The superior graph relates the standard solution concentration with the intensity value obtained from the image. The inferior graph relates the \( \log_2 \) of the solution concentration with the intensity value obtained from the image.

Figure 4.20 Calibration curve obtained averaging the pixel values of the test zone area for the FOB tests. The superior graph relates the standard solution concentration with the intensity value obtained from the image. The inferior graph relates the \( \log_2 \) of the standard solution concentration with the intensity value obtained from the image.

LIST OF TABLES

| Table 3.1 | Samples for HIV Calibration | 65 |
| Table 3.2 | HIV antigen calibration | 67 |
| Table 3.3 | Samples for HIV Batch Testing | 81 |
| Table 3.4 | Final results after test’s fine tuning | 82 |
| Table 3.5 | Samples for HCV Calibration | 82 |
| Table 3.6 | HCV antigen calibration | 82 |
| Table 3.7 | Samples for HCV Batch Testing | 83 |
| Table 3.8 | Final results after test’s fine tuning | 83 |
| Table 3.9 | Sucrose concentration and test performance | 84 |
| Table 3.10 | Anti haemoglobin antibody calibration | 84 |
| Table 3.11 | Anti haemoglobin antibody calibration | 84 |
| Table 3.12 | Samples for Batch Testing | 85 |
| Table 3.13 | Final results for FOB testing | 85 |
| Table 4.1 | Calibration curve AUC of test line regions and ELISA results for the HIV samples | 95 |
| Table 4.2 | Samples for Calibration | 95 |
| Table 4.3 | Calibration curve AUC of test line regions and ELISA results for the HCV samples | 96 |
| Table 4.4 | Samples for HCV Calibration | 97 |
| Table 4.5 | Calibration curve AUC with solution of known concentration for test line regions of FOB test | 98 |
**LFIA**  Lateral flow immunoassay

**POC**  Point of care
Part I

INTRODUCTION
The development of the immunochromatographic test platform is the result of converging technologies. Amongst them the main one is the latex agglutination assay [24]. Although these types of tests have been in the market since the 1970’s, their use became more frequent from the 1990’s until now. During this period, applications for the technology have expanded well beyond clinical diagnostics [22].

Lateral flow immunoassay (LFIA) technology is well stablished, trustworthy and is around for over three decades now. The regular growth of its use with the development of new tests for new parameters comes to demonstrate the large applicability of such technology [26].

For a number of years now, Lateral flow technology (LFT) has been used in commercial tests for the detection of Human immunodeficiency virus (HIV), Hepatites C virus (HCV), Dengue, Chagas amongst other infectious diseases. Most tests make use of colored labels which allow visual test interpretation and simplifies the use of the test device.

LFIA presents as a well-established and appropriate technology when applied to Point of care (POC) testing. The advantages of LFIA are well known and include:

- Mature and established technology;
- Easy to use;
- Easy to manufacture, since the equipments and processes are already developed;
- Can yield high sensitivity and specificity associated with good stability;

Although several advantages may be listed, there are well known limitation to the technique such as:

- Unclear patent situation;
- Performing simultaneous analysis may be difficult;
- Sensitivity may not be sufficient in some systems;

One of the main upsides of the lateral flow test platform is its simplicity which have allowed testing in several different and many times complicated situations [17].

Testing for infectious diseases has always presented a challenge to governments specially in remote, resource sparse areas. The need
of equipments, highly trained personal and a minimal infrastructure which can be costly, has restricted the access to simple methodologies which can diagnose several pathological and clinical situations.

The lateral flow test platform is a simple, usually single step, reliable and can be applied to several test formats. Antigens and antibodies can be detected in the sample and the user needs minimal or no training to perform them.

A commercial test to detect haemoglobin, Fecal occult blood (FOB) can be seen in figure. 1.1. These tests are usually composed of a plastic casing, the test strip which is formed by several membranes overlapped and finally the test buffer which pushes the sample and prepares the medium to react with the colloidal gold or any other label used. In figure 1.1 is presented the test before, during and after reaction run.

![Rapid Test for the detection of haemoglobin in fecal mater prior, during and after the test is ran](image)

Figure 1.1: Rapid Test for the detection of haemoglobin in fecal mater prior, during and after the test is ran

Due to its simplicity and low cost, the lateral flow test platform is now greatly used in the laboratories regular routine, even though it was originally meant for field testing. In an attempt to make HIV and HCV testing more available several governments, specially in those areas where there is very little to no infrastructure, lateral flow immunoassay has become the platform of choice worldwide [19].

In recent year the test for detecting haemoglobin in fecal matter has also came to be very popular. Today it maybe even more used than HIV and HCV. The presence of blood in the stool sample maybe indicative of colon and rectum cancer. In this scenario, a simple detection of occult blood in feces may help early diagnostics and treatment improving the overall patient’s prognostic [32].
As can be perceived, the use of rapid tests can improve the overall health situation of neglected populations and even facilitate screening in laboratories.

Although several advantages can be pointed out in such test platform, there is a large shortcoming of lateral flow technology; which is the lack of quantification methodologies that could yield consistent test results [28].

As previously stated, although very popular and with several advantages, the lack of quantification methodologies and easy to use equipments for such procedure has always been a problem in the LFIA platform. This deficiency causes the technology to suffer in several types of assays in which quantification is of the essence. Also, final result visual interpretation may sometimes be flawed due to the human component, which is always subject to error.

Since its early development, rapid tests have used latex microspheres. With the further development of the nanoparticles chemistry, colloidal gold has become the label of choice for testing. The smaller particle size which yields larger sensitivity, low cost and easy incorporation to the manufacturing process has facilitated the use of the colored nanoparticle. Because of this, the great majority of tests developed and in the market today make use of colloidal gold nanoparticles as revelation reagent [26].

The main problem performing rapid test quantification is that labels which present signals in the visual spectrum have been traditionally used in the test’s platform [6]. These labels impose the necessity of image analysis systems to acquire, treat, select and quantify the information contained in the test strip. For a high level quantification, the overall environmental conditions must also be controlled and the image acquisition method must be reproducible.

The work presented here has the objective of developing three different lateral flow test devices (two for detecting antibodies and one for detecting antigens in the sample) and a method to quantify the final result. The quantification methodology should yield result that provided basis for the construction of a model which should be linear and could be used to calibrate a quantitative test response or to obtain a qualitative ensured result.

The tests developed are comprised of an HIV test, an HCV test and a test which detects Human Hemoglobin in fecal matter. All of the test final results were selected and quantified using image analysis and feature selection techniques. All of the images were obtained using a commercial scanner with a high resolution configuration.

This dissertation is organized as follows. It is commenced by discussing about lateral flow technology itself and possible quantification alternatives in chapter 2 as well as the test’s components and different device settings. This sets a background for the methods used to develop the tests themselves. In chapter 3 it is demonstrated the test’s
development and fine tuning. Section 3.3 demonstrated the development of the HIV, section 3.4 the development of the HCV and section 3.5 the development of the FOB test. In chapter 4 it is demonstrate the image quantification methodology and explore the acquired data to perform quantification for each of the tests. Section 4.3 demonstrates the results obtained with the HIV, Section 4.4 demonstrates de results for the HCV and Section 4.5 demonstrates de results for the FOB. Finally in chapter 5 is analyzed the overall results and the work is concluded. The tests developed by themselves although simple, present a great contribution for their possible impact in people’s everyday lives. Besides that, although we expected that a full quantification method for rapid tests still requires severe control of manufacturing processes, a further exploration of the relation between intensity of the test and control lines correlating with the titer of the analyte contributes to further developing such great test platform.
Part II

BACKGROUND KNOWLEDGE
This chapter reviews most of the significant work on the related tests (HIV, HCV and immunological detection of hemoglobin) and how it was applied in the test’s development besides the image analysis techniques used in the reader system development.

2.1 A LITTLE BACKGROUND INFORMATION

Since this work was intended to join the fields of engineering and biology/immunology, to make the readings more accessible, a short overview of some key concepts and terminologies is performed in the following paragraphs. In fact just a small part of the classical immunology and image analysis is required to better understand the scope of this work.

One of the main points which one must be familiar with to further understand the theory behind the technology is the concept of antibody and antigen. Antibodies are glycoproteins produced by higher vertebrates which have the ability to specifically recognize and bind to a molecular structure in an antigen. Antigens are substances that under the right conditions can induce the production of specific antibodies which bind to them. It is important to highlight that the antibodies bind only to specific portions of the antigen, known as epitopes [34].

Modern immunoassays have been originated from the necessity of detecting complex biological molecules in situations where chemical and physical tests to detect such particles are not suited or simply do not exist. The high sensitivity and specificity of antibody-antigen reactions culminated with the development of several types of test formats using such interaction [37]. Originally immunoassays were developed to allow the study of immunology, specially antibody-antigen interactions. Over time the possibilities of immunoassays were demonstrated, including their ability to work with complex matrices such as human serum and other fluids.

First immunoassays were precipitation based. This technique was followed by radioimmunoassays. Due to its limitations, these methods were gradually replaced by Enzyme linked immunosorbent assay (ELISA). The ELISA method depends on the immobilization of antigens or antibodies onto a solid surface, in the step known as sensibilization. The sample is than added to the sensibilized surface. The analite is captured by the antigen or antibody previously attached. The material that wasn’t captured is posteriorly washed off. Finally an enzyme
coupled to a secondary antibody is added to the reaction surface. A second wash is conducted. The enzyme’s substrate is finally added. If the sample presented the analyte a colored byproduct is formed indicating a positive result. The intensity of the color allows the quantification of the substance of interest [4].

2.2 LATERAL FLOW IMMUNOASSAYS

The lateral flow immunochromatographic test platform, also known as rapid-test, is the result of the combination of several principles and technologies. The technical basis of the lateral flow came from the latex agglutination assay [24]. But it wasn’t until the 80’s that most of the technology needed to further develop this kind of test became available which resulted in the filing of several patents.

The first and main application which derived from the immunochromatographic test platform was the human pregnancy test [33]. Although this was the first test to further develop the lateral flow test platform, several other technologies and production processes needed to mature. Amongst them the most relevant ones were nitrocellulose membrane manufacturing, antibodies and antigens production and purification.

On the next items we will further explore the Lateral Flow platform, its components, formats and overall architecture.

2.2.1 Architecture of a Lateral Flow Immunoassay

The figure 2.1 shows the basic configuration of a Lateral Flow Test. The components include:

- Sample Pad
- Backing
- Conjugate Pad
- Test Line
- Control Line
- Nitrocellulose Membrane
- Absorbent Pad

As can be seen in figure 2.1, the parts overlap amongst them and are mounted on a plastic backing material which gives support and holds the components in place. Each part of the test is made of different materials. When a test is run, the sample is usually added to the sample pad. The sample migrates through the test (this migration can be helped by a buffer). Once the sample reaches the conjugate
pad, it reconstitutes the particles which have been immobilized in this section of the device. These particles usually are colloidal gold or latex microbeads \[36\]. They have been conjugated to one of the assay’s components, which may be an antibody or an antigen. The sample mobilizes the conjugate which interacts with the analyte in sample. The analyte along with the conjugate migrate into the nitrocellulose membrane. As it migrates, they keep on interacting, until the test line is reached. The test line is composed of antibodies or antigens, depending on the test configuration, which capture the complex analyte-conjugate. Excess reagent migrates over the test and control line, being absorbed by the wick. The final result is seen in the nitrocellulose membrane as the presence or absence of lines which captured the conjugate. If colored labels are used, the interpretation may be visual. If labels such as fluorescent particles or paramagnetic monodisperse latex are used, the final result must be acquired by a reader system.

There are two main types of test formats direct sandwich and competitive described below \[22\].

**Direct Sandwich Assay:** Direct assays are typically used when testing for larger analytes with multiple antigenic sites (epitopes). This is the case of HIV, HCV and haemoglobin detection. In this test format, a positive result is indicated by the presence of a test line. On the sandwich assay, some of the target analytes bind to the conjugate and some remain free in solution. When the mixture passes through the capture zone (test line) the marked and unmarked analytes are captured. The free conjugate which did not bind to any analyte, keeps flowing and is captured by the control line, indicating an adequate test’s flow. The control line is usually composed of immobilized antibodies species specific for the antibody in the particle conjugate. This type of test may be seen in figure 2.2.

**Competitive Assay:** Competitive assays are typically used for the detection of small molecules, usually with a single antigenic site which could not bind to two antibodies simultaneously. This format presents a positive result with the absence of a test line. The control line still forms independent of the test line. In this type of format the conjugate binds directly to the control and
test lines without the presence of the analyte. The analyte is not necessary to provide linkage between the test line and the conjugate. In this case when present, the analyte interferes with the binding of the conjugate with the test line. When the solution does not contain any target molecules, the signal is formed at the test line with high intensity. When the solution contains target analytes, the signal is diminished or even not present. This type of test may be seen in figure 2.3

![Diagram of Direct Sandwich Assay Test Configuration](image)

Figure 2.2: Direct Sandwich Assay Test Configuration [22]

### 2.2.2 Commonly Used Materials and Processes in Development and Manufacturing of Lateral Flow Immunoassays

The overall manufacturing process of lateral flow immunoassays is dependent on a series of standard materials. Each material requires further processing from its original (raw) state and each step is essential to obtaining a high quality reproducible test device [9]. A quick review is performed on each component and how it is used on the different tests final assembly. The components and their assembly order are outlined in figure 2.4. The overall process includes mainly:

- Dispensing of test and control lines;
- Membrane blockage (if necessary);
- Conjugate pad pre-treatment;
• Dispensing or dipping of membrane in conjugate;
• Sample pad pre-treatment;
• Assembly of membrane, conjugate pad, sample pad, wick and backing into cards;
• Cards are cut into strips;
• Strips are assembled into cassettes;
• Pouching of cassettes.

### 2.2.2.1 Nitrocellulose Membrane/Analytical Region

The main purpose of the analytical region is to bind protein forming the control and test lines. Besides the initial binding, the membrane must maintain the protein linked or absorbed to its structure. The membrane must also accept the sample and conjugate which flow into it from the sample and conjugate pads. The flow must be consistent and drive the material into the reaction area. The membrane also has to allow the reaction between the sample, conjugate and immobilized proteins to happen.

The membrane has historically been made out of nitrocellulose. Other materials such as nylon and polyvinyllidene fluoride can be
used, but previous attempts to spread their use have not been successful due to a number of issues such as cost, limited utility and lack of knowledge on the material’s further processing.

Nitrocellulose is widely used, and very functional. It possesses certain characteristics that justify it as being the material of choice. Its relative low cost, capillary flow and high protein. Although it has such characteristics nitrocellulose is not the ideal matrix for Lateral flow (LF) tests. Lack of performance reproducibility between lots, high flammability, being subject to scaring and breakage during processing are among the undesirable aspects of the test [25].

In order to function as the reaction matrix in LF assays, the material must be hydrophilic. Nitrocellulose is naturally hydrophobic and it is made hydrophilic during its production process. This is performed by the addition of surfactants. One key point of membranes is that they are porous. These pores are held open by water. If the membrane loses such water, the pores collapse and the membrane loses its wicking ability. This causes flow rate changes and interferes with the test sensitivity leading mainly to false positive results.

The membrane’s capacity to bind proteins occurs through a combination of electrostatic, hydrogen and hydrophobic forces. One of the key elements to obtaining reproducible and sensible assays is the consistent immobilization of immunological active proteins to the test and control lines. It is well know that the proteins lose most of their immunological activity after binding to the nitrocellulose due to its inability to bind covalently or directionally. The accepted model for protein binding is that proteins are initially attracted to the membrane surface by electrostatic attraction and hydrogen and hydrophobic interactions are responsible for long term binding to the membrane. Besides that several other factor may affect protein binding process [18]. Amongst them are:
CHOICE OF REAGENTS: The use of several components in the reagent that is used along with the immunogenic protein may compete or disrupt protein bonding to the nitrocellulose. The use of non-specific proteins such as BSA and casein compete for protein binding sites. Materials which interfere with hydrogen bond such as urea and formamide may also disrupt protein binding to the nitrocellulose. Reagents with the use of materials which interfere with hydrophobic interactions such as tween and tryton may also disrupt protein binding to the nitrocellulose.

PROCESSING METHODS: How the protein is applied to the membrane interferes with how the protein binds or spreads through the membrane. Contact tip versus non-contact will have effects on the line width and how homogenous it is. The drying process is also fundamental. The drying speed, relative humidity and temperature may interfere on the protein final conformation and with it the test sensitivity and specificity.

The final point which must be overviewed on the analytical region theme is that the membranes will suffer several processing methods before integration with the test device. The test and control lines will be deposited, forced air drying and immersion for blocking may be conducted. The membrane will be assembled into cards and cut into strips. All of this processing implicates the necessity of tensile strength and resistance. The use of backed membranes reduces the risk of membrane breakage and damage to its surface and facilitates its handling during production steps [18]. Commercially the membranes are available in rolls or cut to size sheets as can be seen in figure 2.6 and 2.5.

2.2.2.2 The Conjugate Pad

The conjugate pad is used to hold the conjugate and release it efficiently during the test run. Variations in conjugate deposition, drying and release constitute a major cause of variation in assay performance. Due to its most common material (fiber glass) treatment of the conjugate pad previous to conjugate application is necessary. The treatment is usually performed by dipping the material in solution of surfactants, proteins and polymers followed by drying.

The application of the conjugate to the membrane is critical to the process. Two methods are typically used. They are:

- Immersion of the conjugate pad into the conjugate suspension;
- Dispensing with quantitative non-contact dispensers.

An important characteristic of the conjugate pad is that it must not destabilize the conjugate over its shelf-life [30].
The Sample Pad

The ability of accepting several types of samples which can be run in a single step, is a great advantage of lateral flow test devices. The samples spectrum varies from whole blood, urine, serum and several other biological and environmental samples. Because of this wide spectrum of samples, the sample pad presents a great deal of relevance to the test’s proper utilization. The burden of making the samples compatible with the other parts of the test, lies on the Sample Pad. The Sample Pad must efficiently receive the sample, treat it and release the analyte. The sample treatment includes removing red blood cells, adjusting the pH of the sample and bind/remove components of the sample which could compete /interfere with the assay.

Sample pad pretreatment is almost solely constituted of immersion and drying. Specially during drying, loss of homogeneity may be evident due to edge effect. Generally sample pads are constituted of fiber glass, rayon, cellulose or several other materials. One important aspect of the sample pad is its capacity of accepting and withheld the material used in pretreatment and the sample added to the device. The sample pad must help the sample applied to it to be released in a controlled manner, channeling fluids into the assay rather than allowing surface flow or flooding.

During manufacturing the sample pad suffers several manual or automated handling steps. Because of this aspect, tensile strength is an important aspect for the material choosen to integrate the assay. In some cases the conjugate pad and the sample pad can be the same unit, although this is not common [30].
2.2.4 The Wick

The wick is an absorbent material designed to remove the excess fluid from the assay. If the excess fluid is not removed from the reactional matrix, false positive results may occur. In general, the material is constituted of a high-density cellulose [30].

2.2.5 Backing Material

The components of the lateral flow tests are laminated to a backing material which provides structural resistance to the final test strip. The backing material is coated with a pressure sensitive adhesive which holds the various components in place. The backing is in general constituted of polystyrene or other plastic material coated with a medium to high tack adhesive. It is necessary the incorporation of a backing to the test, due to the necessity of laminating several components. The overlapping must be reproducible specially in reader based systems. Variations in overlapping may result in line placement variations and loss of reproducibility in reader based systems [25].

2.2.6 Labels for Detection

The most common particle used for detection in lateral flow device tests is the colloidal gold. Latex monodisperse particles are also very popular. In recent years very sensitive assays using fluorescent and luminescent labels have been developed. These assays which were non-membrane-based, have been adapted into lateral flow formats. Ideally a label for detection in lateral flow presents:

- Simple conjugation, so that biologicals may be conjugated without loss of chemical and biological integrity;
• High stability under several chemical and environmental conditions;
• Simple detection by several methods;
• Presents manufacturing scalability and low cost.

There are many reasons why colloidal gold is the most used label in commercial lateral flow immunoassays. Its manufacturing is easy and well established, the formed color is intense and there is not the necessity of a developing reagent for visualizing it. The label is very stable both in liquid as in dried forms [6].

Colloids are the dispersion of one phase into another. Colloidal gold was discovered to be formed by a elementary gold core surrounded by a negative ionic double layer of charges as can be seen in figure 2.7.

![Colloidal gold molecular structure](image)

Figure 2.7: Colloidal gold molecular structure [35]

The widespread of colloidal gold came about with simpler synthesis methods [10], which were based in gold citrate reduction. These methods produced uniform gold nanoparticles of controlled size. In [16] was firstly reported process of conjugated antibodies into colloidal gold for use in a diagnostic immunoassay. Protein A conjugation has been widely standardized [23], being today the most commonly used label for lateral flow immunoassay.

**Colloidal gold conjugates:** Gold nanoparticles are constituted of an elemental gold particle surrounded by a negative double layer of charges. Because of this structural arrangement, high concentrations of salt (increase in solution’s ionic charge), causes particles flocculation and loss of characteristic red color. The increase of solution’s ionic strength causes the negatively charged
particles to reduce repulsion against each other. With their approximation, they condensate forming large aggregates which is characterized by a blue color development in the solution. Macromolecular ligands adsorb onto colloidal by a combination of electrostatic and hydrophobic interactions [6]. Once the gold is absorbed, or protected by the macromolecular ligand, it is no longer subject to high salt flocculation. This characteristic may be used for determining whether the gold is protected or not after conjugation.

**CoLloidal Gold Formulation:** There are several protocols well established for colloidal gold formulation depending on the particle size desired. Amongst several reducing agents, the most common is by sodium citrate reduction [10]. In this procedure, a mixture of tetrachloroauric acid with deionized water is brought to a boiling point. A sodium citrate solution is added to the gold chloride. Immediately, gold atoms start to form in solution. Its concentration rises rapidly until the solution becomes supersaturated. Particle aggregation occurs following. All the remaining dissolved gold atoms continue to bind at nucleation sites until all atoms are removed from solution. The number of nuclei formed initially determines the number of particles and their size in solution. The more the nuclei, the smallest the final particle size. Some practical aspects of colloidal gold formulation must be attended. Glassware must be very clean and without scratching. All solutions and distilled or dionized water used must be filtered to remove any particles or lint. Sodium citrate should be added to the boiling solution rather than the gold chloride to the citrate.

**Preparation of colloidal gold conjugates:** The conjugation of colloidal gold to macromolecules allows its functionalization. During colloidal gold conjugation, it is important to control the ligand’s and colloidal gold’s pH. Both preparations should be adjusted to pH’s slightly higher than the protein’s isoelectric point. Below the pKi of the ligand, ligand induced flocculation will occur. Above the pKi of the ligand, there is very limited absorption due to charge repulsion between ligand and colloid. The optimal pH for conjugation can be determined by a pH titration series. Series of 0.5mL of colloidal gold suspensions with different pH values (increasing by 0.5) are added to a series of aqueous solutions (50µL) of protein at concentration of 1.0 mg/mL. Flocculation will occur in some of the preparations which is witnessed by a shift from red to gray color. The smallest pH in which flocculation does not occur is the optimal pH for the gold solution stabilization. The minimal amount of protein required for stabilization can also be determined by a concentra-
tion titration. 100µL of colloidal gold in its optimal pH is added to a series of dilutions of the protein also in optimal pH. After mixing, a 10% solution of sodium chloride is added and the formation of flocculation is determined. The minimum amount of protein which prevents flocculation is the minimum amount of protein required to stabilize the gold solution. The minimum amount of protein required to stabilize the gold solution does not represent saturation values. A 10-20% or even a few folds excess can be used to obtain maximum binding. If this is the case, excess ligand must be removed by centrifugation or it would compete with gold-conjugate ligand in the assay [6].

2.3 READER SYSTEMS IN LATERAL FLOW ASSAYS

Readers used in Lateral Flow Assays, specially visually interpreted tests, come to reduce one off the test’s main vulnerability, which is the human component. Since the test is visually interpreted, the final result may be subject to human error during result interpretation. Detection of colloidal gold or other colored labels is performed using CCD devices (charged coupled device). Up to this date very few reader devices have come to light in the market and most of them are used for obtaining qualitative results or semi-quantitative at the most. Lateral Flow Reading Systems have also proven extremely difficult to produce do to lack o test reproducibility and development costs are beyond most small and mid size Lateral Flow Test developers [8].

An important point which must be addressed is that readers do not define the test’s ultimate sensitivity or specificity. The chemistry and biology ultimately provide the result. Considering this aspect, rapid tests manufacturers dedicate most of their efforts into test development and not into reader integrated systems.

2.4 HIV DIAGNOSTIC

As of december 2003, there were approximately 40 million people living with HIV/AIDS worldwide [12]. HIV diagnostic methods have improved deeply the care for patients. They are based on the detection one or more of the molecules which form the HIV virus, or the detection of antibodies produced by infected individuals. Due to these detection methods, is important to further understand the molecules which comprise an HIV particle, before describing the HIV tests.

2.4.1 Structure of HIV

HIV is formed by a capsule composed of proteins, glycoproteins, RNA and fat molecules, which are encapsulated in a viral envelope derived from the membrane of the human’s host cells. Two important
glicoproteins (gp 120 and gp41) extend from the interior of the virus through the viral envelope to the exterior particle. The outer layer of the core is composed of p17 and the inner core of p24 proteins. The core of the HIV holds the HIV genome, which is made up of two single-stranded RNA molecules. The RNA molecules are associated with others proteins needed for HIV propagation and survival such as reverse transcriptase and an integrase which converts the HIV RNA into DNA and integrates the DNA into the host DNA [14].

2.4.2 How HIV infects target cells

The HIV virus binds to CD4 T-helper cells of the immune system using gp120. The HIV fuses with the host cell. The viral RNA and its internal proteins enters the cell, converts the RNA into double-stranded DNA, which is integrated into the host cell, until cellular events trigger its activation. With the HIV activation, viral particles are released from the host cell. The cellular damage from the expulsion of the viral particles causes the infected cell to die.

2.4.3 Different HIV subtypes

There are two main HIV subtypes that can cause aids, HIV-1 and HIV-2. The great majority of AIDS cases are caused by HIV-1. HIV-1 frequently mutates and it is a highly genetically diverse virus. HIV-1 has been classified into two groups, groups M and O. This classification is made on the different sequences of viral envelope.

2.4.4 Serological profile of HIV infection

There are three well characterized stages in HIV infection. The first is the acute phase immediately after HIV infection. This phase is know as serological window. In this stage the p24 antigen and viral nucleic acid are detectable but antibodies are not. In this stage the host is seronegative and HIV RNA levels in blood spike at about 6 weeks and than decline. CD4 T-cells count drops rapidly until about 6 weeks post infection.

After a 6 to 8 weeks period antibodies start to be detected in the host. Antibody detection is known as serumconversion and is the beginning of HIV second phase of infection. This is the chronic phase. During this phase the immune response against the virus evolves and grows. During several years the antibody population matures. CD4 T cell count slowly declines. The final phase of HIV infection also know as AIDS, is marked by the surging of clinical symptoms. This phase is marked by immunodeficiency and contraction of microbial opportunistic infections and levels of CD4 T cell lower than 200 cell/mm³.

2.4.5 HIV Diagnostic Tests

HIV diagnostic tests detect either host antibodies against different HIV proteins or by detecting different components of the virus such as HIV RNA or p24 antigen. The most common form of test is serological. In it, the viral type, period of infection (long lasting or recent) and even viral load may be determined.

2.4.5.1 Tests which detect host antibody specific to the virus

Several assay types detect antibodies against and/or particles of the virus. Amongst them are enzyme based immunoassay, Western blot, immunofluorescent assays, rapid tests, urine tests, saliva tests and detuned assays. Since this work focusses in rapid test, a further overview will be performed in this item.

**Rapid tests for HIV detection:** Enzyme based immunoassays are cost-effective and time-efficient when processing a large number of samples. Although they require trained personal and specific laboratory equipment. Rapid HIV tests are used in great part in situations where tests results must be provided immediately and in smaller labs which handle a small number of samples for triage. Rapid tests also do not require highly trained personal to be conducted. Several rapid assay formats exist; amongst them the most common is the lateral flow based assay. In this type of assays, the specimen is absorbed into a pad and immediately combined with a signal reagent. The specimen-signal reagent migrates through the reaction matrix (nitrocelullose membrane) and if the test is positive, a line appears on the membrane [5].

2.5 Hepatites C Diagnostic

Hepatitis C is a disease that affects 200 million people globally. HCV is an extremely dangerous pathogen both because it’s high prevalence and potentially serious complication of persistent HCV infection. Chronicle cases of the disease may lead to cirrhosis, hepatocellular carcinoma and end stage liver disease. One of the main aspects of HCV is that it has a high rate of mutation which enables the virus to scape the organism’s immune response. Major HCV genotypes genotypes constitute genotype 1,2,3,4,5 and 6 [27].

2.5.1 Structure of HCV

HCV genome has a single stranded RNA molecule. This molecule codes a glycoprotein of 3000 amino acids. The protein transcripted by the genetic material and post processed by viral and cellular proteins
yield 10 mature proteins. Among those proteins is the core, which form the nucleocapsid, and the envelope glicoproteins E1 and E2.

2.5.2 How HCV infects target cells and replicates

The first step in the infectious cycle, is the attachment of the virus to the host cell. There is specific interaction between a receptor on the host cell surface and a viral attachment particle. The protein CD81 was identified as the HCV receptor. It interacts strongly with viral E2 protein. Besides this entry route, HCV may also enter the cell by binding to LDL protein receptor. The viral protein E1 is involved in the membrane fusion. Once inside the cytoplasm, the genomic RNA is directly translated. Particle formation may be initiated by the core proteins interacting with the RNA genome. Viral nucleocapsid acquire their envelopes by budding though the endoplasmatic reticulum membranes. In this case the virus may be exported via constitutive secretory pathway.

2.5.3 HCV Diagnostic Tests

HIV diagnostic tests detect either host antibodies against different HIV proteins or by detecting different components of the virus such as HIV RNA or p24 antigen. The most common form of test is serological. In it, the viral type, period of infection (long lasting or recent) and even viral load may be determined [3].

2.5.3.1 Tests which detect host antibody specific to the virus

Several assay types detect antibodies against and/or particles of the virus. Amongst them are enzyme based immunoassay, Western blot, immunofluorescent assays, rapid tests, urine tests, saliva tests and detuned assays. Since this work focusses in rapid test, a further overview will be performed in this item.

Rapid tests for HCV detection: Enzyme based immunoassays are cost-effective and time-efficient when processing a large number of samples. Although they require trained personal and specific laboratory equipment. Rapid HCV tests are used in great part in situations where tests results must be provided immediately and in smaller labs for triage. Several rapid assay formats exist amongst them the most common is the lateral flow based assay. In this type of assays, the specimen is absorbed into a pad and immediately combined with a signal reagent. The specimen-signal reagent migrates though the reaction matrix (nitrocellulose membrane) and if the test is positive, a line appears on the membrane [15].
2.6 IMMUNOLOGICAL DETECTION OF HAEMOGLOBIN IN FECAL MATTER

The detection of colorectal cancer in asymptomatic patients has been accepted as the most effective way of improving the disease’s prognosis. Undoubtedly, colonoscopy is the gold standard in the diagnosis of colorectal cancers. Although this is the ideal exam, the risk of bowel perforation and bleeding, not considering the patient’s discomfort during examination, leads to low compliance in screening. Because of this, less invasive methodologies of detecting lesions are applied. These tests are based on the detection of occult blood in stool specimens.

Normally 0.5 to 1.5 mL of blood is lost is stool, amounting 2mg of haemoglobin per gram of stool. This amount is typically not detected by occult blood tests. These tests which detect bleeding in the colon rectal portion of the intestine can be grouped into Guaiac based tests, heme-porphyrin tests and immunochemical tests [32].

2.6.1 Guaiac based tests

Guaiac-based tests detect the pseudo-peroxidase activity of heme either as intact haemoglobin or as heme. This activity converts colorless guaiac to a blue color in the presence of an oxygen donor such as hydrogen peroxide in the developing reagent. The probability that a guaiac-based test will be positive is directly related to amount of fecal heme, which is related to the size and location of the bleeding lesion. In general guaiac based tests perform better in detecting larger and more distal lesions. One of the problems in this test is that haemoglobin levels must exceed 10mg/gram of stool (equivalent to the lossage of 10mL/day of blood) for this kind of test be positive. Although physiologically, individuals may present up to 1mg of haemoglobin per gram of stool material, nonspecific positive results may also occur. The main set back in the kind of test is the need of dietary restriction, since the ingestion of foods or medication with heme group in them may cause false positive results. Several aspects as the ingestion of vitamin C, improper sampling and haemoglobin degradation by colonic bacteria may cause false negative results [11].

2.6.2 Heme Porphyrin tests

The tests based in the heme porphyrin methodology, determine Haemoglobin (Hb) derived spectrofluorometrically which allows exact measurement of total haemoglobin in stool. The advantage of this test compared to guaiac based methodology, is that the presence of vegetable peroxidase and rehydration do not affect the results. But in the same manner as the guaiac test, the presence of non human
haemoglobin may cause false positive results, which requires dietary restriction previous to the test.

2.6.3 Immunochemical tests

This methodology detects intact or nearly intact globin groups. Since the globin group of humans is different from other species, the test is specific in detecting human haemoglobin which removes the need of dietary restrictions previous to testing. Theoretically this technique is more specific to lower intestine blood loss, because blood from lower sites is less degraded during intestinal transit. This methodology makes use of antibodies against globin epitopes. They can detect very low levels of blood added to stool. One of the test’s main shortcomings is the loss of globin antigenicity at room temperature.

2.6.4 Interpretation of occult blood tests

In general a positive result in occult blood tests is not indicative of disease. There are several inherent errors in specimen collection and storage. Besides that, small amounts of blood in stool is physiological. Larger amounts, which allows detection by immunological test, can happen due to several other reasons other than colon cancer. Several factor can affect the detection of occult blood in stool such as anatomical level of lesion, stool transit time, stool mixing, intraluminal haemoglobin degradation and intrinsic features of bleeding of gastrointestinal lesion.

In screening studies from 2 to 16% of patients presented positive results using this type of test. In individuals with physiological blood loss, without indication of other pathologies, the tests presented 20% of false positive results. Although commercial tests should present similar results, large differences in sensitivity and specificity can be identified.

2.7 Quantification of lateral flow device tests

As previously discussed, early diagnosis of infectious diseases is of the essence to prevent long term effects of emerging public health threats and to reduce the disease’s burden by avoiding infection by other individuals. Besides that, early diagnosis in several diseases may just as well be the key factor between cure or a quality life and several complication due to the disease’s stage.

Rapid diagnosis tests provide a cost effective and practical tool to screen large groups of individuals in a laboratory environment or in the field. In parallel to the RDT (Rapid Diagnostic Technology) development, the current status of global communication, exhibits a promising potential to be used for healthcare [20].
Several works are being conducted in the realm of using cell phone’s cameras to obtain image from the test devices. With more than 5 billion subscription worldwide, the cell phone can be utilized for imaging, sensing, processing and distributing health related data even in field settings. Most developed reading/quantification techniques makes use of devices adapted to the cell phone, in which the test device is inserted and read from. Such device for smart phones can be seen in figure 2.8

Figure 2.8: (a - b) Different views of a RDT reader prototype installed on an Android phone (Samsung Galaxy S II). This attachment can be repeatedly attached/detached to the cellphone body without the need for fine alignment and modification. (c - d) Schematic diagrams of the designed optical RDT reader attachment are shown. Depending on the format of the diagnostic test, users can switch between the two illumination schemes (reflection vs. transmission) to acquire an image of the RDT with high contrast. This image is then rapidly processed within less than 0.2 sec/image through a custom-developed application running on the cellphon to generate an automated report that consists of test validation and reading of the diagnostic results as well as quantification of the test line’s color intensities [20].

Over the last few years there has been a considerable effort focused toward the development of digital test readers which are capable of semi or quantitative RDT analysis. As a result several commercial products have been introduced in the market, including LFRDR101 Reader, ESEQuant Lateral Flow System, RDS-1000 and DOA Test Reader. However these existing test readers are still bulky and costly. They also read only a limited number of RDTs from a given manufac-
This limits their wide scale use and forces the user to work only with a determined brand of tests.

2.7.1 Image Analysis Techniques for Test Quantification

Digital image processing allows one to enhance image features of interest while attenuating detail irrelevant to a given application, and then extract useful information about the scene from the enhanced image [29]. Images are produced by a variety of physical devices, including still and video cameras, x-ray devices, electron microscopes, radar, and ultrasound, and used for a variety of purposes, including entertainment, medical, business (e.g. documents), industrial, military, civil (e.g. traffic), security, and scientific. The goal in each case is for an observer, human or machine, to extract useful information about the scene being imaged. Once an image is digitalized, it can be processed by several operations. Image processing operations can be divided into three major categories:

A. Image Compression;
B. Image Enhancement and Restoration;
C. Measurement Extraction.

Often the raw image is not directly suitable for the purpose of extracting useful information from the scene, and must be processed in some way. Such processing is called image enhancement. Processing by an observer to extract information is called image analysis. Enhancement and analysis are distinguished by their output, images vs. scene information, and by the challenges faced and methods employed. Image enhancement has been done by chemical, optical, and electronic means, while analysis has been done mostly by humans and electronically.

Digital image processing is a subset of the electronic domain wherein the image is converted to an array of small integers, called pixels, representing a physical quantity such as scene radiance, stored in a digital memory, and processed by computer or other digital hardware. Digital image processing, either as enhancement for human observers or performing autonomous analysis, offers advantages in cost, speed, and flexibility, and with the rapidly falling price and rising performance of personal computers it has become the dominant method in use.

Image defects caused by digitalization process, faults in the image set-up such as bad lighting, can be corrected using image enhancing techniques. Once the image is in good conditions, it can be used to obtain important information by means of measurement extraction operations.

An image may be defined as a two-dimensional function $f(x, y)$, where $x$ and $y$ are spatial coordinates and the amplitude of $f$ at any
pair of coordinates is the intensity. Color images are formed by the combination of individual 2-D images. In the RGB color system, a color image consists of three (red, green, blue) individual color component images.

An image function may be represented as the following:

\[
F = \begin{bmatrix}
    f(1,1) & f(1,2) & \cdots & f(1,N) \\
    f(2,1) & f(2,2) & \cdots & f(2,N) \\
    \vdots & \vdots & \ddots & \vdots \\
    f(M,1) & f(M,2) & \cdots & f(M,N)
\end{bmatrix}
\]

where \( M \) is the number of lines and \( N \) the number of columns.

Image based test readers need to extract relevant spatial test features and use such information embedded in grayscale pixel intensity of each rapid test device image. Test readers initially convert colored image to grayscale. After such conversion, the areas relative to test and control lines are clipped from the image. The average column pixel per row is obtained. This provides a column vector in which each element in the vector is the average value of the pixels in the corresponding row of the grayscale image. In this vector several features may be used and are. Some systems make use of the maximal value of the average column. Every pixel less than 90% of this value is zeroed in order to remove parts of the image which do not carry useful information. This leaves only non-zero values for pixels that are part of the rapid test strip itself. Other systems discard 20% of the columns taken from both ends to avoid spatial artefacts. Then the absolute value of the derivative of the average column pixel intensity per row vector is calculated to locate the rows that the strip lies on. The acceleration in pixel values occur right before and right after the target flow area. Once the flow rates are obtained, they are processed to get location of the test and control lines. The local maxima of the row vector indicate the position of the lines.

This overview on rapid test place the ground for next chapters in which are presented the device development and the test’s quantification methods.
Part III

DEVELOPED WORK
3 TEST DEVELOPMENT

In this chapter is present the development of the HIV, HCV and FOB test devices as well as their performance against positive and negative samples.

3.1 RAPID TEST DEVELOPMENT

As previously stated, the development of the rapid immunochromatographic test strip, was the result of the convergence of several threads which can be traced back to the 1950’s. Only in the 1970’s though, the basic principles of lateral flow technology were established [4].

The actual modern assay is composed of several parts that overlap into each other and are mounted into support sheets. When a test is run, sample is usually added to the proximal end of the strip. Also known as sample pad. In this portion, the sample is usually treated so it can become compatible with the remainder of the test. The now treated sample migrates through to the conjugate pad where it reconstitutes the immobilized colloidal gold particles (or other label). The immobilized particles have been conjugated to one of the specific biological components of the assay which can be an antigen, an antibody or other proteins, depending on the assay format. The analyte in the sample interacts with the reconstituted conjugate and migrates into the reaction matrix (the nitrocellulose membrane), in which the other specific biological component are immobilized. Antigens, antibodies or other proteins are laid down in bands in specific areas of the membrane where they capture the analyte and the conjugate as they migrate by the capture lines [17].

During the course of this work three tests were developed and adjusted using the basic principles of lateral flow technology. Two of them HIV and HCV are antibody detection tests and one of them, the FOB, is an antigen detection test.

For each test, a primary format was stablished and posterior adjustments were performed to improve its performance. The adjustment phase was crucial and required extensive testing to ensure the device’s final performance.

The primary format for the antigen detection test was the usual lateral flow. The colloidal gold functionalized was freeze dried and assembled to the conjugate pad. One of the antibodies was immobilized at the membrane and the other was conjugated to the gold. An antibody species specific was used at the control line. The antibody detection tests had the specific antigen immobilized at the membrane.
The colloidal gold was conjugated to protein A and kept in liquid format. A dilution/transport buffer was added to the conjugated colloidal gold to facilitate its flow through the membrane.

Antibody detection tests have historically presented larger complications. The sample usually presents a large amount of proteins which can interact with the test’s components and cause incorrect results. Antigen detection tests are less complicated since the antibody interaction with the antigen in the sample is more specific. Problems regarding false positive and false negative results in this test format present less of a challenge to be solved.

### 3.2 Test Development Steps

The test development can be broken down in several steps. Amongst them are the test format selection, proteins prospection and selection, label production, protein concentration adjustment, membrane blocking, pilot lot production and final quality control testing. Those steps can be seen in figure 3.1. The most crucial step in the test’s development is protein selection. If this step is not carefully performed, even with great deals of efforts, the end result may not be satisfactory in terms of sensitivity, specificity and overall performance. Each one of the steps is further detailed below:

![Diagram of test development steps]

**Figure 3.1:** Lateral flow test device development steps
**Test Format Selection:** Several test formats can be chosen depending on the tested analyte. As stated previously, the most common for point of care testing are the lateral-flow and flow through formats. In this work we used lateral flow test formats. The manufacturing facility, reduced cost, high through output facilitates its use and production wise becomes a more accessible product. The HIV and HCV tests were based on a sequential assay format. In this test format, the sample interacts with the conjugate initially. The antibodies in the sample marked by the conjugate are captured in the test line by the immobilized antigen. If the sample presents the antibody against the antigen immobilized in the test line, the colored label is captured in that region and the test presents a visual positive result. The control line indicates if the assay has worked properly and is constituted of protein A immobilized in it. Antibodies in the sample marked by the colored particles are captured in the control region by the protein A previously immobilized. The FOB test is a sandwich type of assay in which a pair of antibodies specific for the analyte are conjugated to the colloidal gold and immobilized to the nitrocellulose membrane. If present in the sample, the analyte is captured by the colloidal gold conjugated to one of the antibody’s pair. The complex analyte-conjugated migrates though the membrane and is captured by the immobilized antibody in the test region. The control line is constituted by an antibody specific to the Fc portion of the antibody conjugated to the colloidal gold.

**Proteins Selection:** Although the physical components of the lateral flow test strip, construction techniques, buffers and surfactants play a major role in optimizing the test, at the very heart of the tests are the biologicals. They are the major responsible for the high sensitivity and specificity in assays. For the HIV and HCV the proteins were acquired at Meridian LifeScience. Other source of antigens were identified, but with the short time and large scope of this work, the selection of well known and extensively tested antigens by other parties was preferred, specially due to the necessity of standardizing so many other aspects of the test platform itself. Another key aspect which was taken into account when using Meridian’s antigens, is that smaller companies tend to fluctuate in quality, material concentration and even availability of the proteins in time. A larger supplier tends to reduce such problems and minimize the necessity of having to restandardize the entire test. The FOB antibodies used came from Medix Lifescience. This is a well know antibody manufacturer and this antibody pair has also been extensively tested and is used in several know tests in the market.
**Label Production:** As stated previously several labels are available to be used in lateral and dynamic flow tests. The most common ones are latex beads and colloidal gold nanoparticles, but colloidal carbon, gold nanorods, colloidal platin amongst others are also available options. In this work we choose colloidal gold due to its preparation easiness, well know and described behavior, low cost and high sensitivity. Due to its smaller size and possibility of packing a larger amount of particles in an area, makes colloidal gold a more sensible label when compared to latex microbeeds. The protocol used is a modification from [10], in which gold chloride is reduced by citrate addition under ebullition. Although there are several vendors for concentrated colloidal gold, we choose to synthesize it to incorporate a gold chloride reduction method in this work. The facility of manufacturing such label under mild conditions creates the opportunity to incorporate as many productions steps as possible in the test manufacturing process.

**Protein Concentration Adjustment:** There are two main protein adjustment procedures which will be described in detail in this work. The first and perhaps most important one is adjusting the protein conditions and concentration for conjugation onto colloidal gold. The second is the protein adjustment for adsorption in the reactional matrix. This procedure involves titrating the protein to ensure that the overall test presents a high sensitivity without compromising it’s specificity. The protein conjugation to colloidal gold involves several steps. A resumed procedure is dialyzing the protein to reduce or remove any salt from solution. High ionic strengths in solution causes the gold nanoparticles to coalesce and flocculate. In order to be conjugated, the protein’s pH has to be adjusted slightly below its isoelectric point. This guarantees optimal conjugation. One important issue is the amount of protein that stabilizes the gold. The minimum amount of protein is established performing serial dilutions and conjugating such solutions. After the gold is stable, adding salt to the solution does not cause it to flocculate. If the gold is not properly protected by the protein, it will flocculate changing from a red, burgundy color to a bluish gray color. Excess protein may be used to protect the gold nanoparticles although excess free protein may compete with the conjugated protein which reduces the test’s sensitivity. The second protein adjustment is the amount of protein applied to the reactional matrix, the nitrocellulose membrane. Excess protein may cause false positive results due to non specific interactions with sample and conjugate. Too little protein may cause low signal which may not be visually detected. The right amount of protein must be carefully determined and added to the test.
MEMBRANE BLOCKING: Membrane blocking to prevent nonspecific binding of the detector particle and analyte is not absolutely essential to obtain a functional lateral flow immunoassay test strip. Depending on the test format, antibody, antigen, sample system sometimes the use of blocking agents is required to guarantee the functionality of some tests. Several strategies may be applied to adding the blocking agent. Perhaps the easiest and still effective method is adding the blocking agent to the sample pad. Once the sample is added the blocking agent co-migrates with the sample along the strip. Another option is applying the blocking agent directly to the membrane by dipping the membrane into a reservoir of blocking solution. The concentration and type of blocking agent must be determined empirically for compatibility with the sample and antibody system. Excess blocking agent may dry down as crystals which occlude the membrane’s pores. A final washing step in buffer alone may be necessary to remove excess. Applying blocking agents may improve flow characteristics, although is not necessary to all test formats. The most common materials used are tween-20, triton x-100, PVA, PVP, PEG, Brij and BSA. Such material may be used isolated or combined.

PILOT LOT PRODUCTION: After all conditions are defined and initial tests have shown the desirable performance (usually against a small set of samples) a small production lot is prepared. Such lot is made mimicking all production steps and operations. If possible it should be handled by production personal. This lot is usually presents a size of 30% of a regular production lot.

QUALITY CONTROL: Rapid test production presents several steps and all of them must be carefully controlled. Creating controlling procedures for each step of production is as important as the test development itself. Some steps are essential in production. Amongst them are membrane performance testing, impregnated membrane performance testing, conjugated colloidal gold optical density measurement, conjugated colloidal gold activity, and finally, final test performance against know characterized positive and negative samples. The final testing guarantees that the chance of false positive and negative results are minimized. One key step during manufacturing is adjusting the protein concentration when the lot of antigen or the membrane is altered. This is a key point because the variation in the membrane pores causes the sensitivity of the test to vary and each lot of protein usually comes in slightly different concentrations.
3.3 HIV Detection Test

3.3.1 Test Format Selection

The HIV test development can be broken down in several steps. The first step is test format selection. As stated before several test formats are available in the literature. The two most common ones are the Flow-through and the Lateral Flow. The Flow-through usually makes use of Protein A conjugated to the colloidal gold and the antigen immobilized to the membrane. This test format is becoming less and less popular although it is a reliable and well known format. The usual test procedure for this kind of assay requires the sample to be added to the device. If the sample presents antibodies against the HIV for instance, those antibodies will bind to the antigen immobilized in the membrane. The manufacturing complexity in spotting individual membranes has made the Lateral Flow format cheaper and more popular. Some other aspects have also made the use of the Lateral Flow format more abundant such as the possibility of using whole blood samples in a single step.

The proposed test format in this work fuses the flow through with the lateral-flow techniques. A lateral flow strip impregnated with the HIV antigen is used. The conjugate is a Protein A colloidal gold. The main difference of the lateral flow technique is that the colloidal gold is kept liquid and receives a surfactant that resembles the washing buffer usually applied in the flow-through test format. This test format brings the versatility of a universal conjugate with the high sensitivity and manufacturing strong points of the lateral flow platform.

3.3.2 HIV Antigen Selection

The cern of an optimal antibody detection test is the use of highly specific antigens. Such proteins must present high specificity and also must have highly immunogenic epitopes so most infected patients present antibodies against the specific portion of the infectious agent. For sequential assay types, the solubility of the protein in buffer without urea or detergents is not crucial. If the protein must be conjugated to colloidal gold, must be kept in mind that, high concentrations of ions in solution can cause the gold solution to flocculate.

There are several vendors of HIV antigens in the market. Those vendors offer several antigens which possess various portions of the HIV fused together or isolated portions of it. A well known and already tested antigen in several test formats is a product available by Meridian Life Science. This antigen is composed by an envelope portion of HIV-1 virus, conjugated to the gp36, also an HIV-1 protein. A peptide from gp36 HIV-2 is also fused to the chimeric protein. It also contains the C-Terminal portion of the GP120 and most of gp41
proteins. To complete the antigen, a 23 peptide section derived from pg36 of HIV-2 is also added. This protein has already been tested in several test formats such as Western Blot, Lateral Flow and Elisa.

### 3.3.3 Label Production

The colloidal gold production uses a simple procedure which is a variation from [10]. A 1% gold chloride solution is prepared. One gram of gold chloride from sigma had it’s bottle carefully cleaned and the label removed. The bottle was weighed and then opened. 5mL of water were added to the flask which was closed and vigorously shaken. All the liquid was removed from the flask. Another 5mL were added to guarantee that there were no residues of gold chloride inside the flask. The flask was left to dry and than weighted again. Deionized water was added to the gold solution until it reached the concentration of 1%. This procedure was carefully done to guarantee the right gold concentration which affects the colloidal gold particle size. A second solution of sodium citrate 4% in water was prepared using high grade reagent also from sigma. Both solutions were stored in dark glass bottles.

New glassware was carefully cleaned with soft tissue and mild detergent. The glassware was rinsed with deionized water carefully to remove any traces of detergent. The glassware was than boiled in deionized water to guarantee the removal of any detergent traces in it. The material was left to dry upside down to avoid any particles to be caught in its interior.

One liter of deionized water was added to a two liters erlenmeyer. This water was brought to a boil using a hot plate. With the water boiling, 20mL of the gold chloride solution was added inside the glassware. The gold chloride solution was left heating. When the solution started to boil intensively, 20mL of the sodium citrate solution was added integrally to erlenmeyer. The new solution was vigorously stirred for 30 seconds and left under heating again. Every minute the solution was removed from the hot plate and stirred for about 10 seconds. Immediately after the citrate solution was added, the gold chloride solution changed from a mild yellow color to transparent and then started to darken. It became light gray, blue and changed into an intensive red color as can be seen in figure 3.2. After the solution changed into its characteristic color, it was left under heating for another 10 minutes. In about 5 minutes it changed to a mild burgundy color with an orange tone to it when held against light. The orange tone in the background indicates that the gold nanoparticles present a size of about 15nm. No particle size measurement was performed since this synthesis is well characterized and the macroscopic characteristics of it demonstrated that the solution was "healthy". After the synthesis is complete the material was left to cool down with a lid to protect it.
from particles which may cause the gold solution to flocculate. After the solution cooled down, distilled water was added bringing the volume back to 1L.

![Figure 3.2: Gold nanoparticles macroscopic appearance after synthesis](image)

3.3.4 Protein Concentration Adjustment

Protein concentration adjustments can be divided into two different operations. Both of them are as important. The first is the protein conjugation to the colloidal gold and the second adjusting the amount of antigen impregnated to the membrane.

3.3.4.1 Protein Conjugation to Colloidal Gold

Colloidal gold is a very unstable solution before being conjugated to protein or other molecule which avoids the nanoparticles to collide and flocculate. Two different protocols may be used to conjugate the colloidal gold to protein or other specific macromolecules. The easiest method is by adding an excess protein to the solution in a low concentration. By these means all particles are equally covered by the protein. The main problem with such a procedure is that the excess protein in solution which does not conjugate to the gold will compete with the conjugated material for the analyte in solution. This may reduce a test’s sensitivity and the solution may have to be centrifuged...
to remove the supernatant and with it, any non conjugated protein. The problem with this procedure is that there is a large lossage of gold nanoparticles during centrifugation which is also a costly procedure. The most logical fashion to conjugate protein to colloidal gold is by determining the minimum amount of protein which stabilizes the gold solution.

The best conjugation protocol adapted during the course of this work for protein A, the selected protein for the developing reagent, was by titrating a protein A solution. A 1 mg/mL protein A with 0.09% azide solution was prepared from lyophilized protein A in powder from G.E.. A dilution series was prepared in a microplate. 50µL of dionized water was added to each microwell. 50µL of the protein A solution was added to the first microwell. 50 µL of this solution, from the first microwell was added to the second and 50µL from this added to the next microwell. This was done until the last microwell. On the last one, 50 µL of solution was discarded. After that, 50µL of the colloidal gold solution was added to each microwell. The conjugation is instantaneous and if the colloidal gold is protected, it becomes stable even at a high ionic charge solution. After waiting 5 minutes once the gold was added on the dilution series, 50µL of a 10% sodium chloride solution was added to each microwell. Where there was not enough protein to protect the gold, once the sodium chloride was added, the color immediately changed from its characteristic red, to an intense blue, as can be seen in figure 3.3.

The first well that presented stabilized gold, indicates the minimum amount of protein that stabilizes a certain amount of this gold solution. The image presented in figure 3.3 shows that 1mL of the protein solution stabilizes 128mL of gold. This indicates that to stabilize 1L of gold, it is needed 7,8mL of the protein solution. As several conjugations were performed, was noticed that if the protein concentration added to the gold is larger than 0,5 mg/mL, the gold solution some times flocculates. To avoid that, the protein solution is further diluted in water before being added to the gold to a final concentration of approximately 0,2 mg/mL.

To perform the conjugation, 8 mL of the protein solution 1,0 mg/mL was diluted in water until it reached a concentration of 0,2 mg/mL. Under intense agitation, the new, low concentration protein solution was integrally added to the gold. This new solution was than left sitting for 15 minutes under refrigeration. A small sample of it (100 µL) was taken and mixed with 100µL of a 10% sodium chloride solution. Since the color did not change, this demonstrated that the gold solution became stable.

To further protect the stabilized gold, 50mL of an albumine 20% (protease free from sigma) and azide 1% solution was added. The albumin blocks the gold nanoparticles and protects it over time. The azide works as a strong preservative and guarantees that there won’t
be bacterial growth in solution. After the albumin solution was added the material was left sitting for another 15 minutes. This guarantees that any unconjugated gold left in solution is coated by protein. This is important because if the solution starts to flocculate it may loose overall stability and also activity.

The final step in the colloidal gold synthesis and functionalization is pH control. For long term storage in liquid format, ideally, protein A should be kept in a pH of 7.4 and under physiological conditions in terms of sodium chloride. The best alternative for that, since the functional colloidal gold is now resistant to higher ionic charges in solution is adding a concentrated PBS buffer in solution. A 10 times concentrated PBS buffer with high sodium chloride and azide was added to solution. This buffer is constituted of 7.8 grams of NaH₂PO₄, 17.91 grams of Na₂HPO₄ − 12H₂O, 90 grams of NaCl and 10 grams of NaN₃ per liter of solution. This buffer solution is added to the conjugated gold solution in a proportion of 100mL per liter of protein A gold. This new protein A - gold is now active, functional and stable.

Figure 3.3: Determination of protein needed to stabilize gold

3.3.4.2 Improving Flow Characteristics of Protein A Gold

The regular lateral flow device test, uses the functional colloidal gold which is added to a conjugate pad. When running the test, the dried and immobilized colloidal gold is reconstituted by the sample or sample/buffer. It than reacts with the analyte in the sample. The conjugate was kept in its liquid form so it is easier to adjust. Maintaining the conjugate in this format reduces several steps during production which may reduce manufacturing costs and labor in the future.

The main problem identified keeping the gold in liquid format, is that since it does not have surfactants in it, the flow characteristics once it is added to the membrane are not ideal. The conjugate’s flow speed is very small and the flow is uneven. Another point detected was that the conjugate in this raw form presented an extremely high activity
which favors false positive test results. A simple solution to improve flow characteristics and reducing the activity was to add a surfactant filled buffer to the protein A gold. It was identified that this buffer needed to be constituted of several important components which favors the test’s behavior. Several tests have been made with several known buffers and surfactant formulations in several proportions. It was identified that the buffer added to the Protein A gold should present tween-20, albumin, heparin and tris buffer.

Each component of this solution possesses a specific characteristic that acts to improve the protein A gold’s overall characteristics, which improves the test’s performance.

**TRIS BUFFER**: An important characteristic of the protein A gold, is the pH range. In order to preserve the protein A stability and overall characteristics, a mild buffer is used in this solution to maintain the final’s solution pH. Tris buffer was selected because a high phosphate concentration was previously used in the protein A gold and more phosphate in solution could tamper with the overall final stability due to the high ionic strength in solution.

**ALBUMIN**: Albumin is an excellent blocking agent. Its use guarantees the protein A gold stabilization. It also dampens the protein A gold extra activity avoiding non specific interaction between the gold and the immobilized antigen.

**TWEEN-20**: Tween-20 is used in solution to ensure the test’s wetability. This mild detergent ensures that the the sample and colloidal gold will flow correctly throught the membrane. The molecular structure of the detergent guarantees that the components in the sample and conjugate solution can correctly interact to give optimal results.

**HEPARIN**: Heparin has been added to the buffer to avoid/reduce possible false positive or negative results. Many times, samples may present micro blood clots which can interfere in the sample and conjugate flow. They can be responsible for causing false positive and negative results. The heparin added breaks such possible clots and reduces the possibility of flow interference and incorrect results.

### 3.3.4.3 Control Line Protein Adjustment

Much different from the test line adjustment, the control line concentration adjustment is much simpler. Since protein A is being used in the revealing reagent, protein A was also added to the control line.

Excess antibodies which do not bind at the test line are captured in the control region. The protein A gold than links to this antibody revealing the control line. This indicates that enough protein A gold
ran through the strip so any result, positive or negative may be consid-
ered reliable. A concentration which was found to be ideal, presenting
good results was 1.0 mg/mL of protein A in 0.1% azide. After impreg-
nation, both the control and test lines were left to dry under controlled
humidity (below 30% for 24 hours). The impregnation was performed
simultaneously (both the test and control lines).

3.3.4.4 Test Line Protein Titration

Perhaps the most challenging and key aspect of developing lateral
flow device tests is adjusting the amount of protein added to the
reaction matrix. If too much protein is added, non specific interaction
which results in massive false positive results occur. If too little protein
is added, false positive results may not be entirely cleared, but false
negative in low titer samples start to appear. To ensure full protein
absorption to the reactional matrix, after impregnation, the membranes
were left to dry for 24 hours under controlled humidity (below 30%).
Adjusting such aspect of the test is key to guarantee optimal test
performance. Also several adjuvants may be added or not to the
system in order to improve test performance, enhance sensitivity or
reduce non specific interaction. the most commons are:

Sucrose: During the drying stage of manufacturing, water is re-
moved from the reaction matrix and only the protein immobi-
lized is left. If all water is removed from the protein, it might
loose its conformation resulting in loss of activity. The sucrose
preserves water in the system, reducing the loss of protein’s
three dimensional conformation.

Albumin: Albumin is an excellent blocking agent. It may be used
directly alongside with the active protein to reduce non specific
interaction. This reduces false positive results, but it may also
reduce the protein’s activity and cause false negative results.

Azide: Azide is a well known and stablished preservative. It’s use
avoids microbiological growth and preserves the protein immo-
ibilized in the test and control lines.

3.3.4.5 Tests Performed and Optimization

In this section we specifically detail the concentration adjustments,
testing procedure and optimization of all test components. To perform
test optimization, a set of samples was selected according to table
3.1. A known set of positive and negative samples allows an initial
adjustment of the test before testing it against larger sets of samples.
These samples were carefully chosen to allow as optimal as possible
initial calibration, reducing the need of rework.

Another important aspect which was defined, and from it all other
adjustments to the test were performed, is usage protocol for the test
Listing 3.1: Testing Procedure for HIV Test Device

1 - Place all materials and components on top of flat clean surface;
2 - Add 2 drops (100 µL) of colloidal gold to reagent port in the plastic cassette;
3 - Wait until the reagent front passes the sample area;
4 - Add 5µL of sample to the sample port (serum or plasma);
5 - Read the results with 10 minutes;
6 - If a line in the test region and a line in the control region appears, the test is positive;
7 - If a line in the control region appears, the test is negative;
8 - If no lines appear or if only the test line appear, the test is invalid.

The first component to adjust is the dilution buffer for the protein A gold. A well known formulation which is used in flow-through tests is a mixture of albumin 10g/L, tween-20 5 ml/L, heparin 50 Units/L and...
sodium chloride 0.9% w/v. This buffer constitution is traditionally used to wash the protein A gold used in flow-through tests. This buffer presents several characteristics which are interesting for the test’s format such as improving flow characteristic and allowing the nanoparticles to reach and leave the test and control line regions.

This buffer was added directly to the protein A gold in a 1:1 ratio. This gave us the final revelation solution. During the test’s development, some false positive results were present in negative samples. Such behavior was improved by adding another 3% salt to the revelation solution. The salt reduces the nonspecific interaction between the antibody and the antigen immobilized in the nitrocellulose.

Since the revelation reagent and testing procedure were standardized as previously stated, the amount of antigen added to the nitrocellulose membrane needed to be determined. Excess antigen may result in non specific interaction, but too little antigen may cause a very week signal which may be interpreted as a negative result. To perform such calibration. The positive and negative samples were tested against several antigen concentrations according to table 3.2. The antigens were diluted into a sucrose 5% and azide 0.1% solution. The sucrose was added to help the protein to keep its three dimensional structure when it is dried in the membrane. Two different concentrations gave primarily satisfiable results as can be seen in table 3.2. They are 0.0625 and 0.03125 mg/mL. In order to reduce costs, but still maintaining the test’s performance, the lower concentration was kept as the initial standard. Must be pointed that each test was run in triplicate and the results presented were reproducible.

The antigens were added by spotting to the cut membranes. About 1µL of solution was added to each strip. This method for adding material to the membrane is just a means of calibrating the test. Once the concentrations are adjusted, the test and control lines are added to the test strips by a specialized equipment, in this case, the Autokun ® sprayer.

After the concentration for the test and control line’s were adjusted a small batch of about 300 tests was manufactured. These tests were manufactured using a commercial sprayer and guillotine cutter. They followed a full manufacturing process. The equipments used are from the Autokun ® company as can be seen in figure 3.4 and 3.5. The test strips were manufactured maintaining the standards obtained during test calibration. The strips were cut to fit in the plastic cassettes. In this case, with 4.5 mm.

Once the batch was manufactured the strips were tested against a larger set of know characterized samples. These samples included serum from several known positive and negative patients and are presented in table 3.3, in the column identified as "First tests". Testing the finalized strips against this material guarantees that the test will perform against several different interferents which may be present.
Table 3.2: HIV antigen calibration

<table>
<thead>
<tr>
<th>Sample/[Ag.]</th>
<th>1.0</th>
<th>0.5</th>
<th>0.25</th>
<th>0.125</th>
<th>0.0625</th>
<th>0.03125</th>
<th>0.0156</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+ 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HIV+ 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>HIV+ 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>HIV+ 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>HIV- 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

As can be seen in the column "First tests" of table 3.3, the initial test calibration allowed the test strips to present enough antigen immobilized which guaranteed enough sensitivity but not an excess which could cause massive false positive results. A certain number of false positives is expected in this phase of the development. A well known and usual artifice is membrane blocking and salting the conjugate to reduce non specific interaction. Both of them were performed. A blocking protocol which consists of treating the membranes with PEG 30000 daltons followed by membrane blocking with albumin was applied. An extra 3% sodium chloride was also added to the conjugate. This alone removed 50% of the false positive results. After the test and control lines were added, the master sheets were dipped in a 0.5% solution of PEG 30000. The membranes were left to dry under controlled humidity, below 30% for 24 hours. This cleared the remainder of the false positive results, and did not interfere with the true positive ones as can be seen in last column of table 3.3, "Second tests".

After the final fine tuning of the test parameters, another batch of 700 tests was manufactured following the conditions stated before. The strips were cut to size and assembled. A batch of 100 known characterized negative and 25 known positive samples were run each sample in triplicate. The results can be seen in table 3.4. The ROC curve in figure 3.6 demonstrated the performance of this binary classifier, in this case, the HIV testing device.
Following the development procedures used for the HIV, the HCV test development can also be broken down in several steps. The first step is test format selection. Like in the HIV, the proposed test format fuses the flow through with the lateral-flow techniques. A lateral flow strip impregnated with the HCV antigen is used. The conjugate is also Protein A colloidal gold.

### 3.4.2 HCV Antigen Selection

As stated for the HIV, the cern of an optimal antibody detection test is the use of highly specific antigens. Such proteins must present high specificity and also must have highly immunogenic epitopes so most infected patients present antibodies against the specific portion of the infectious agent. There are several vendors of HCV antigens in the market. Several antigens possess various portions of the HCV fused together and others are isolated portions of it. A well known and tested antigen in several test formats is a product available by Meridian Life Science. This antigen is composed by Hepatitis C Virus (HCV) Nucleocapsid NS3/NS4/NS5 recombinant. It also contains GST fusion partner. The total molecular weight is of 70.8 kDa. This protein has already been tested in Lateral Flow and Elisa test platforms.
3.4.3 Label Production

The colloidal gold used for the HCV test is the same as the one for the HIV. The same procedure stated in subsection 3.3.3 is used to manufacture the gold used. The final result after the synthesis is shown in figure 3.2. The procedure to synthesize the gold nanoparticles presented excellent results for the HIV, and it was reproduced for the HCV test. In order to universalize the conjugate used, the same protein A gold is used for both the HIV and HCV test.

3.4.4 Protein Concentration Adjustment

The protein concentration adjustments can be divided into two different operations. Both of them are as important. The first is the protein conjugation to the colloidal gold and the second adjusting the amount of antigen impregnated to the membrane.

3.4.4.1 Protein Conjugation to Colloidal Gold

In the HCV test, as it is for the HIV, the samples are researched for antibodies against the pathogen. Since in essence those tests are similar,
the same colloidal gold conjugated to protein A is used for both of them. The same procedures stated in subsections 3.3.4.1 and 3.3.4.2 can then be repeated.

3.4.4.2 Control and Test Line Protein adjustment

The control line adjustment has already been performed in subsection 3.3.4.3 for the HIV test. This protein concentration can be extrapolated for the HCV or any other antibody detection test. The concentration used for the control line in the HCV is then 1,0mg/mL of protein A in a 0,1% azide solution.

The test line adjustment for the HCV is as challenging as the one previously reported for the HIV. In the same manner, if too much protein is added, non specific interaction can result in an unacceptable amount of false positive results. If too little protein is added, false negative in low titer samples may start to appear.

The procedure of leaving the proteins to dry under controlled humidity is repeated for the membranes in the HCV test, as it was done for the HIV.

3.4.4.3 Tests Performed and Optimization

In this section all the concentration adjustments and optimization performed for the HCV test are detailed. Similar to the HIV test in subsection 3.3.4.5. To perform test optimization, a set of samples was also selected according to table 3.5 to calibrate the test. This known
Listing 3.2: Testing Procedure for HCV Test Device

1. Place all materials and components on top of a flat clean surface;
2. Add 2 drops (100 uL) of colloidal gold to the reagent port in the plastic cassette;
3. Wait until the reagent front passes the sample area;
4. Add 5uL of sample to the sample port (serum or plasma);
5. Read the results with 10 minutes;
6. If a line in the test region and a line in the control region appears, the test is positive;
7. If a line in the control region appears, the test is negative;
8. If no lines appear or if only the test line appear, the test is invalid.

A set of positive and negative samples allows the initial adjustment of the test before testing it against larger sets of samples.

Another important aspect which was also defined, and from it all other adjustments to the test were performed, is the usage protocol for the test device. The protocol is the same as the one used for the HIV test and is detailed in Listing 3.2.

As previously, the first component to be adjusted is the dilution buffer for the protein A gold. The same dilution buffer used for the HIV was used for the HCV. This buffer improves the flow and has presented satisfactory results for the HIV test. This buffer was also added directly to the protein A gold in a 1:1 ratio. This gave us the final revelation solution. In the HIV test an extra 3% of sodium chloride was added to conjugate to reduce the false positive result rate. To universalize the conjugate for both tests, this was also done for the protein A gold used in the HCV test.

With the standardization of the revelation reagent and test procedure, the amount of antigen added to the nitrocellulose membrane needed to be determined. To perform the calibration, the positive and negative samples were tested against several antigen concentrations according to Table 3.6. The antigens were diluted into sucrose 5% and azide 0.1% solution. The sucrose was added to help the protein to keep its three dimensional structure when it is dried in the membrane. One concentration gave primarily satisfactory results as can be seen in Table 3.6. It is 0.0625 mg/mL. For the HCV as well as for the HIV each test was run in triplicate and the results presented were reproducible.

The antigens were also added by spotting to the cut membranes. About 1uL of solution was added to each strip.

After the concentration for the test and control line’s were adjusted a small batch of about 300 tests was manufactured. These tests were produced also using Autokun ® sprayer and cutter. During the process
it was maintained the standards obtained during test calibration. With the strips ready, they were tested against a larger set of known characterized samples. These samples included several known positive and negative samples and they are presented in table 3.7.

As can be seen in table 3.7, the initial test calibration allowed the test strips to present enough antigen immobilized which guaranteed enough sensitivity and specificity. Since no false positive nor false negatives were detected in this phase of the development, there was no need for membrane blocking, as was done for the HIV test. This significantly reduces manufacturing time and cost. Another batch of 700 tests was then manufactured following the conditions stated before. The strips were cut to size and assembled. A batch of 100 known characterized negative and 25 known positive samples were run each sample in triplicate. The results can be seen in table 3.8. The ROC curve in figure 3.7 demonstrated the performance of this binary classifier, in this case, the HCV testing device.

![Figure 3.7: ROC curves of batch testing using finalized test devices](image)

3.5 FOB DETECTION TEST

3.5.1 Test Format Selection

In the same manner as the HIV and HCV test developments, the FOB development was broken down in several steps. As before, the first step is test format selection. Since the FOB test detects the globin portion of the human haemoglobin, it is an antigen detection test.
Because of this, the test selected was also the lateral flow format. Antigen detection tests, make use of a pair of antibodies to make a sandwich with the antigen in the sample. One of them is immobilized to the reaction matrix and the other one is conjugated to the colloidal gold or any other used labels. Once the sample is added to the test, if the antigen is present, the colloidal gold - antibody, binds to the antigen. The complex antigen - antibody - colloidal gold migrates through the nitrocellulose membrane until it reaches the other anti globin antibody which has been immobilized to the membrane in the test region.

The proposed format for this test is the regular lateral flow. In this format, the gold is added to the conjugate pad and dried. Once the sample is added, the solution in which the sample is present reconstitutes the nanoparticles. The reconstituted colloidal gold reacts with the sample as it migrates through the test until it reaches the test and control regions. One main difference of this test with others, which usually make use of serum, plasma or whole blood is that since the sample is solid or semi-solid and a specific antigen is being searched in this material, the sample must be "opened". It was used a buffer with detergents, which facilitate the sample "opening" and also improves the flow characteristics of the membrane.

3.5.2 FOB Antibody Selection

In the same manner as the antibody detection tests, the cern of and optimal antigen detection test is the use of a high quality antibody pair. Such antibodies must be highly specific to human globin portion of the haemoglobin. Cross reaction with the globin of other species, specially those present in regular feeding habits may cause false positive results.

Medix Biotech has a well known monoclonal antibody pair which is widely used by several manufacturers. This antibody pair presents high specificity and sensitivity against human globin protein. Each of the antibodies is specifically design to bind to a different portion of the globin increasing the pair’s sensitivity.

There are two options to lay the control line. Protein A or Goat anti-mouse Fc can be used. Both of them bind to the Fc portion of the monoclonal antibody used. The anti-mouse Fc antibody was selected for this application.

3.5.3 Label Production

The colloidal gold production uses very similar procedure as the one used for the HIV and HCV tests. This synthesis still is a simple procedure which is a variation from (FRENS PROCEDURE). The same gold chloride %1 solution and sodium citrate %4 previously prepared was used. Also new glassware was carefully cleaned as
previously stated. The main difference between this gold solution and the previous used, is that historically low sensitivity is a problem with antigen detection tests. Due to this track history, this gold solution is more concentrated than the ones used for the HIV and HCV. The problem of using high concentration gold, is that it easily flocculates. Extra care was taken inspecting the glassware for scratches and during the washing procedures.

Similarly to the previous used protocol, one liter of deionized water was added to a two liters erlenmeyer. This water was brought to a boil using a hot plate. With the water boiling, 25mL of the gold chloride solution was added inside the glassware. The gold chloride solution was left heating. When the solution started to boil intensively, 25mL of the sodium citrate solution was added integrally to erlenmeyer. The new solution was vigorously stirred for 30 seconds and left under heating again. Every minute the solution was removed from the hot plate and stirred for about 10 seconds. As before, immediately after the citrate solution was added, the gold chloride solution changed from a mild yellow color to transparent and then started to darken. It became light gray, blue and changed into a intensive red color. Unlike the previous gold nanoparticles solution, this formulation presented a much more intense color due to the higher number of nanoparticles present. As before after the solution changed into its characteristic color, it was left under heating for another 10 minutes. In about 5 minutes it changed to a strong burgundy color with an orange tone to it when held against light. The orange tone in the background indicates that the gold nanoparticles present a size of about 15nm. After the synthesis is complete the material was left to cool down with a lid to protect it from particles which may cause the gold solution to flocculate. After the solution cooled down, distilled water was added bringing the volume back to 1L. This solution was left resting under refrigeration for 24 hours protected from light to ensure that it would not flocculate.

3.5.4 Protein Concentration Adjustment

The protein concentration adjustments as before can be divided into two different operations. The first is the antibody conjugation to the colloidal gold and the second one is the adjustment of the amount of antibody impregnated to the membrane. Since both proteins are antibodies, one of them must be conjugated to the gold and the other added to the membrane. The antibody manufacturer usually indicates which one should be conjugated, and which should be impregnated. This was also the case. If the antibody pair is new and not extensively tested, both of them should be conjugated and impregnated. Then the best configuration can be selected.
3.5.4.1 Antibody Conjugation to Colloidal Gold

As previously stated, colloidal gold is a very unstable solution before being conjugated to protein or other molecule which avoids the nanoparticles to collide and flocculate. Since antibodies have a high cost, the best protocol to conjugate them is by determining the minimum amount of antibody which stabilizes the gold solution. There are some extremely important differences between conjugating antibodies and protein A which must be pointed out. The best conjugation protocol adapted during the course of this work for antibodies was by titrating minimum salt antibody solutions in conjugation optimal pH gold.

The original antibody solutions come in stabilization buffers which are usually non compatible with conjugation. Because of this, the antibody was diluted to a 1 mg/mL concentration with 20 mM Hepes, 0.1% azide solution with a 7.4 pH carefully adjusted. This antibody solution was left under dialysis for 24 hours with one exchange of dialysis buffer. The dialysis buffer is also a 20 mM Hepes, 0.1% azide solution with a 7.4 pH carefully adjusted.

After the antibody was dialyzed, it was titrated in the same manner as the protein A for the antibody detection tests. A dilution series was prepared in a microplate. 50µL of 20mM Hepes without azide freshly prepared was added to each microwell; 50µL of the dialyzed antibody were added to the first microwell; 50 µL of this solution, from the first microwell were added to the second and 50µL from this added to the next microwell. This was done until the last microwell. On the last one, 50µL of solution were discarded. After that, 10% of a 200 mM Hepes, pH 7.4, without azide were added to the unconjugated gold solution. This brings the gold solution’s pH to 7.4 which is ideal for antibody conjugation. This new unconjugated gold with controlled pH was then used; 50µL of this gold were added to each one of the previous microwells.

The first well that presented stabilized gold, indicated the minimum amount of antibody that stabilizes a certain amount of this gold solution. Similar to the situation presented in figure 3.3, 1mL of the antibody solution stabilized 64mL of gold. This indicates that to stabilize 1L of gold solution, are needed 15,63mL of this solution. Performing several conjugations with antibodies, was noticed that if the protein concentration added to the gold is larger than 0.1 mg/mL, the gold solution some times flocculates. To avoid that, the protein solution is also dissolved in azide free, 20mM newly prepared hepes, before being added to the gold in a concentration of 0.1mg/mL.

To perform the conjugation, 3.9 mL of the dialyzed antibody solution 1.0 mg/mL were dissolved in hepes 20mM until it reached a concentration of 0.1 mg/mL. Under intense agitation, the protein solution was integrally added to 250mL of the gold solution (pH adjusted gold). The gold solution was than left sitting for 15 minutes under
refrigeration. A small sample of it (100 µL) was taken and received 100 µL of a 10% sodium chloride solution. Since the color did not change, this demonstrated that the gold solution became stable.

To further protect the stabilized gold, 12.5 mL of an albumin 20% (protease free from sigma) and azide 1% solution were added. Similarly to the protein A gold solution, the albumin blocks the gold nanoparticles and protects it over time. The azide works as a strong preservative and guarantees that there won’t be bacterial growth in the solution. After the albumin solution was added the material was left sitting for another 15 minutes. This guarantees that any unconjugated gold left in solution is coated by protein.

The final step in this colloidal gold synthesis and functionalization with antibodies is also pH control. Since the functionalized colloidal gold is now resistant to higher ionic charges in solution adding a concentrated PBS buffer in solution guarantees the pH control of the solution. A 10 times concentrated PBS buffer with high sodium chloride and azide was added to solution. This buffer is constituted of 7.8 grams of NaH$_2$PO$_4$, 17.91 grams of Na$_2$HPO$_4 - 12$H$_2$O, 90 grams of NaCl and 10 grams of NaN$_3$ per liter of solution. This buffer solution is added to the conjugated gold solution. This new antibody-gold is now active, functionalized and stable.

### 3.5.4.2 Adding Colloidal Gold to the Test

Since the chosen format is the regular lateral flow device test, it uses the functional colloidal gold which is added to a conjugate pad. When running the test, the dried and immobilized colloidal gold is reconstituted by the sample added to the buffer, and reacts with the analyte in the sample.

There are several ways for adding the conjugate to the conjugate pad. The most common one is spraying the conjugate to the membrane and air drying the material under high temperature and controlled humidity. To better preserve the conjugate, the material was frozen and freeze dried for 48 hours. The freeze drying process when compared to air improves the flow characteristics of the test, although in terms of sensitivity, usually the results are practically the same.

Some special care was taken during the manufacturing process, since after the material is freeze dried, it can absorb humidity very easily which may result in loss of activity very rapidly. After the material was removed from the freeze dryer, it was kept under low humidity at all times.

The colloidal gold was added to fiber glass membranes by dipping the membrane in a solution batch. The excess material was removed by letting it drip for about 5 minutes. The sheets were then placed in stainless steel trays and frozen for 24 hours at -40°C. After the material was frozen, it was removed from the freezer and immediately placed
inside the freeze drier, as can be seen in figure 3.8. The overall process takes from 48 to 56 hours to be finished.

Figure 3.8: Tray freezedryer from Liotop ®

A key component of the test is the sucrose which must be added to the colloidal gold before the material is impregnated. It acts as a cryopreservative, protecting the proteins during the freezing process. Several concentrations were tested, The ideal one which gave ideal flow was 2%. At higher concentrations, when reconstituted, the material presented a syrup consistence which caused the functional gold to flow very slowly. Low sucrose concentrations, caused the test to run faster, but the material became less active. No sucrose caused the colloidal gold to practically loose its activity. This can be seen in table 3.9. During assembly, 4 by 8 millimeter sections were used per test strip.

3.5.4.3 Control Line Protein Adjustment

The control line in this test format is made out of species specific antibody. A 1,0 mg/mL antibody, 5% sucrose in 1XPBS buffer with pH 7.4 and azide 0,1% was used. This antibody concentration when matched with the prepared colloidal gold presented an easily visualized, high signal control line. This can be seen in figure 3.9. Although lower concentrations of antibodies, presented similar results, since the cost
of the control line antibody is low, an excess of it guarantees that the test will perform accordingly.

![Figure 3.9: Lateral flow strip with test and control lines](image)

3.5.4.4 **Test Line Protein Titration**

In the same manner as the tests which detect antibody in the samples, this test format also requires adjusting the amount of protein, in this case antibody, added to the reaction matrix. If too much protein is added the sensitivity of the test can become too great, and since many individuals may present physiological amounts of blood in feces, the test may give false positive results to patients which do not present significant amounts of blood in their stool sample. If too little antibody is added, the sensitivity of the test may become too small, and the test may present false negative results.

Most of the commercial tests in the market present a sensitivity of 50 ng of haemoglobin per mL of transport solution. The test line was calibrated to detect levels of haemoglobin of 50 ng/mL. To perform such calibration, solutions of 25 ng/mL, 40 ng/mL, 50 ng/mL, 75 ng/mL and 100 ng/mL were prepared from human haemoglobin from Sigma. The table 3.10 shows the response of the test to the different concentrations amounts of haemoglobin in solution. With that calibration, the selected antibody concentration was 0.13 mg/mL.

The same system for the control line is used for the test line. The final antibodies solutions presented 5% sucrose in 1XPBS buffer with pH 7.4 and azide 0.1%. All proteins impregnated in the membranes were left drying for 24 hours under controlled humidity, below 30%. Only after 24 hours the strips were used. Leaving the material to dry
under controlled humidity guaranteed that they presented optimal sensitivity.

3.5.4.5 Transport Solution

A key aspect for the FOB test is "opening" the sample. The test needs to extract any residues of haemoglobin from fecal mater. To do that a tube is inserted in the fresh stool sample and inserted in the transport buffer as is detailed in listing 3.3. This solution must extract the haemoglobin molecules from the sample, preserve these molecules from degradation and once added to the test, reconstitute the colloidal gold and favor liquid migration through the sample.

Traditionally the transport solutions presents only buffer in water for pH control to avoid haemoglobin degradation. Although that formulation was attempted, with the chosen membrane, the material flow was too slow. To improve the test’s flow and reduce reading time, a solutions of tween-20 1.5%, 1XPBS, with pH of 7.4 and azide 0.1% was used. The addition of tween-20 considerably improved test’s flow speed and overall performance.

3.5.4.6 Tests Performed and Optimization

In this section we specifically detail the fine adjustments and optimization of all test components. To perform the FOB’s optimization, a set of samples was selected according to table 3.11. Since the test was previously calibrated with hemoglobin solutions, the main point of this test battery is assuring that the test will behave as expected against previously tested samples.

An important aspect which was also defined, similar to what was done for the HIV and HCV, and from it all other adjustments to the test were performed, is the usage protocol for the test device. The protocol is different from those determined for the HIV and HCV and is detailed in listing 3.3.

This set of known positive and negative samples allows any possible needed adjustment of the test before testing it against larger sets of samples. These samples were carefully chosen to allow the verification of the tests performance against other commercially available tests.

As can be seen from table 3.11 the developed test performed similarly to the leading test in the market. No adjustments seemed necessary. With this information a larger set of samples was used to test the developed device as can be seen in table 3.12.

As expected from previous results, the test performed as expected with real samples. No fine tuning was needed in this test, since the calibration with the haemoglobin solutions guaranteed optimal test performance. Strong and weak signals in the commercially available test was reproduced in the developed device. After this batch testing a new batch of 700 tests was manufactured following the conditions
Listing 3.3: Testing Procedure for FOB Test Device

1 - Place all materials and components on top of flat clean surface;
2 - Insert the sample collection tube in the sample, making sure all areas of the sample are covered;
3 - Vigorously shake the collection tube;
4 - Add four drops of the collection solution to the sample port;
5 - Read the results with 10 minutes;
6 - If a line in the test region and a line in the control region appears, the test is positive;
7 - If a line in the control region appears, the test is negative;
8 - If no lines appear or if only the test line appear, the test is invalid.

stated before. The strips were cut to size and assembled. 100 known characterized negative and 25 known positive samples were used in the newly manufactured tests. Each sample was run in triplicate. The results can be seen in table 3.13.

Figure 3.10: ROC curves of batch testing using finalized test devices
Table 3.3: Samples for HIV Batch Testing

<table>
<thead>
<tr>
<th>Classification</th>
<th>LF result</th>
<th>ELISA result</th>
<th>First tests</th>
<th>Second tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+ 1</td>
<td>+</td>
<td>234 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 2</td>
<td>+</td>
<td>423 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 3</td>
<td>+</td>
<td>19 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 4</td>
<td>+</td>
<td>177 (+)</td>
<td>+</td>
<td>+</td>
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<td>HIV+ 5</td>
<td>+</td>
<td>209 (+)</td>
<td>+</td>
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<td>+</td>
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<td>HIV+ 7</td>
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<td>172 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 8</td>
<td>+</td>
<td>144 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 9</td>
<td>+</td>
<td>273 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 10</td>
<td>+</td>
<td>209 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 11</td>
<td>+</td>
<td>331 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 12</td>
<td>+</td>
<td>107 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 13</td>
<td>+</td>
<td>204 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 14</td>
<td>+</td>
<td>389 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV+ 15</td>
<td>+</td>
<td>256 (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIV- 1</td>
<td>-</td>
<td>0,3 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 2</td>
<td>-</td>
<td>0,2 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 3</td>
<td>-</td>
<td>0,8 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 4</td>
<td>-</td>
<td>0,6 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 5</td>
<td>-</td>
<td>0,3 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 6</td>
<td>-</td>
<td>0,4 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 7</td>
<td>-</td>
<td>0,7 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 8</td>
<td>-</td>
<td>0,7 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 9</td>
<td>-</td>
<td>0,5 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 10</td>
<td>-</td>
<td>0,8 (-)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 11</td>
<td>-</td>
<td>0,2 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 12</td>
<td>-</td>
<td>0,7 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 13</td>
<td>-</td>
<td>0,6 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 14</td>
<td>-</td>
<td>0,3 (-)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIV- 15</td>
<td>-</td>
<td>0,3 (-)</td>
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<td>-</td>
</tr>
</tbody>
</table>
Table 3.4: Final results after test’s fine tuning

<table>
<thead>
<tr>
<th>Positive Sample</th>
<th>Negative Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Result</td>
<td>25</td>
</tr>
<tr>
<td>Negative Result</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.5: Samples for HCV Calibration

<table>
<thead>
<tr>
<th>Classification</th>
<th>LF result</th>
<th>ELISA result</th>
<th>Reference Lab Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV+ 1</td>
<td>+</td>
<td>12 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 2</td>
<td>+</td>
<td>16 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 3</td>
<td>+</td>
<td>19 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 4</td>
<td>+</td>
<td>8 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 5</td>
<td>+</td>
<td>4 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV- 1</td>
<td>-</td>
<td>0,3 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 2</td>
<td>-</td>
<td>0,5 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 3</td>
<td>-</td>
<td>0,7 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 4</td>
<td>-</td>
<td>0,1 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 5</td>
<td>-</td>
<td>0,1 (-)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.6: HCV antigen calibration

<table>
<thead>
<tr>
<th>Sample/[Ag.]</th>
<th>1,0</th>
<th>0,5</th>
<th>0,25</th>
<th>0,125</th>
<th>0,0625</th>
<th>0,03125</th>
<th>0,0156</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV+ 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCV+ 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 3.7: Samples for HCV Batch Testing

<table>
<thead>
<tr>
<th>Classification</th>
<th>LF result</th>
<th>ELISA result</th>
<th>Test’s Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV+ 1</td>
<td>+</td>
<td>12 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 2</td>
<td>+</td>
<td>16 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 3</td>
<td>+</td>
<td>19 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 4</td>
<td>+</td>
<td>8 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 5</td>
<td>+</td>
<td>4 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 6</td>
<td>+</td>
<td>7 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 7</td>
<td>+</td>
<td>12 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 8</td>
<td>+</td>
<td>11 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 9</td>
<td>+</td>
<td>5 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 10</td>
<td>+</td>
<td>14 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 11</td>
<td>+</td>
<td>17 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 12</td>
<td>+</td>
<td>4 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 13</td>
<td>+</td>
<td>3 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 14</td>
<td>+</td>
<td>7 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV+ 15</td>
<td>+</td>
<td>9 (+)</td>
<td>+</td>
</tr>
<tr>
<td>HCV- 1</td>
<td>-</td>
<td>0.3 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 2</td>
<td>-</td>
<td>0.5 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 3</td>
<td>-</td>
<td>0.7 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 4</td>
<td>-</td>
<td>0.1 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 5</td>
<td>-</td>
<td>0.1 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 6</td>
<td>-</td>
<td>0.3 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 7</td>
<td>-</td>
<td>0.4 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 8</td>
<td>-</td>
<td>0.2 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 9</td>
<td>-</td>
<td>0.3 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 10</td>
<td>-</td>
<td>0.5 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 11</td>
<td>-</td>
<td>0.6 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 12</td>
<td>-</td>
<td>0.3 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 13</td>
<td>-</td>
<td>0.2 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 14</td>
<td>-</td>
<td>0.2 (-)</td>
<td>-</td>
</tr>
<tr>
<td>HCV- 15</td>
<td>-</td>
<td>0.4 (-)</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3.8: Final results after test’s fine tuning

<table>
<thead>
<tr>
<th>Positive Sample</th>
<th>Negative Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Result</td>
<td>25</td>
</tr>
<tr>
<td>Negative Result</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.9: Sucrose concentration and test performance

<table>
<thead>
<tr>
<th>[Sucrose]</th>
<th>Flow time (s)</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0%</td>
<td>30</td>
<td>Low</td>
</tr>
<tr>
<td>0.5%</td>
<td>36</td>
<td>Low</td>
</tr>
<tr>
<td>1.0%</td>
<td>44</td>
<td>Normal</td>
</tr>
<tr>
<td>1.5%</td>
<td>52</td>
<td>Normal</td>
</tr>
<tr>
<td>2.0%</td>
<td>68</td>
<td>Normal</td>
</tr>
<tr>
<td>2.5%</td>
<td>75</td>
<td>High</td>
</tr>
<tr>
<td>3.0%</td>
<td>83</td>
<td>High</td>
</tr>
<tr>
<td>3.5%</td>
<td>89</td>
<td>High</td>
</tr>
<tr>
<td>4.0%</td>
<td>94</td>
<td>High</td>
</tr>
<tr>
<td>5.0%</td>
<td>103</td>
<td>High</td>
</tr>
</tbody>
</table>

Table 3.10: Anti haemoglobin antibody calibration

<table>
<thead>
<tr>
<th>[Antibody]</th>
<th>100 ng/mL</th>
<th>75 ng/mL</th>
<th>50 ng/mL</th>
<th>40 ng/mL</th>
<th>25 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 mg/mL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.50 mg/mL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.25 mg/mL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.13 mg/mL</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.06mg/mL</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.11: Anti haemoglobin antibody calibration

<table>
<thead>
<tr>
<th>Classification</th>
<th>LF result</th>
<th>Test’s Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOB 1+ 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 2+ 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 3+ 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 4+ 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 5+ 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 1- 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB 2- 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB 3- 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB 4- 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB 5- 1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 3.12: Samples for Batch Testing

<table>
<thead>
<tr>
<th>Classification</th>
<th>LF result</th>
<th>Test’s Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOB 1+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 2+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 3+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 4+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB 5+1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+11</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+12</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB+13</td>
<td>+</td>
<td>+</td>
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<td>FOB+14</td>
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<td>FOB+15</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOB-1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-5</td>
<td>-</td>
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</tr>
<tr>
<td>FOB-6</td>
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<td>-</td>
</tr>
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<td>FOB-7</td>
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<td>-</td>
</tr>
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<td>FOB-8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-10</td>
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<td>FOB-11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-12</td>
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<td>-</td>
</tr>
<tr>
<td>FOB-13</td>
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<td>-</td>
</tr>
<tr>
<td>FOB-14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FOB-15</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3.13: Final results for FOB testing

<table>
<thead>
<tr>
<th></th>
<th>Positive Sample</th>
<th>Negative Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Result</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>Negative Result</td>
<td>0</td>
<td>98</td>
</tr>
</tbody>
</table>
RAPID TEST QUANTIFICATION

In this chapter is present the quantification methods as well as obtained results quantifying the HIV, HCV and FOB tests previously developed.

4.1 CHALLENGES REGARDING TEST QUANTIFICATION

Rapid test quantification has always been a challenge for test developers. Very rarely exist such a broad spectrum of competencies during test creation and standardization, in order to develop the devices keeping in mind the need of its quantification as an end result.

Lateral flow immunoassays have been around for over 30 years and it is remarkable that only in recent years, reading methodologies have started to be exploited. To improve lateral flow immunoassay’s sensitivity, some companies have replaced colloidal gold beads with fluorescent dyes and paramagnetic particles. Since those labels cannot be detected by the naked eye, such technologies created demands for the development of readers for quantitative analysis [20].

As previously stated there are several colored labels which can be used. The commonly applied to tests are colloidal gold and latex microbeads. Since gold particles are smaller more particles can occupy the same area than latex microbeads. Because of that tests using them can reach higher levels of sensitivity. The advantage of latex microbees is the possible use of several different colors, since there are several commercially available colors for them and colloidal gold presents one basic color.

Since this work is focused in developing tests which can be used with or without a reader system, the used label was colloidal gold as can be seen in chapter 3. Due to the use of colored particles, the quantification method requires the use of image acquisition and analysis. Most of the work concerning readers which operate in the visual spectrum, makes use of CCD based imaging systems. A simple easy access device for labs mainly are table scanners. These devices present low cost and high durability. Since rapid tests have been adopted by most laboratories as a screening method, before using more costly procedures such as CLIA and Elisa, the proposed reader uses a high definition table scanner to perform image acquisition.

To obtain quantitative results, several factors and design issues must be taken into account to assure an accurate test outcome. The figure 4.1 shows a typical intensity profile of a regular rapid-test. The lower valley is the control line intensity; the second valley is the test line...
intensity. The "X" axis is the position of the test and control lines in the strip, and the "Y" axis is the test and control lines intensity measurement.

Figure 4.1: Intensity profile of a test’s acquired image

There are several known errors which have to be addressed to be avoided, guaranteeing reproducible quantifiable readings [8].

4.1.1 Positioning Errors

There are three different positioning errors which can deeply interfere in the test’s quantification final outcome. The first is the relative positioning of the test and control bands during printing. These bands can be closer or farther away from each other or be shifted in relation to the strip’s end sections. The second positioning error is cutting of the membranes, resulting in slightly different lengths. The third positioning error is the placing of the membrane strip into the plastic cassette. The strips can be positioned a little bit to the right or to the left, relative to the reading window and the cassette housing. All these three errors if added can add up to a large error. As can be seen in figure 4.1 slight positioning errors may cause the strip to be read at a point which is outside the maximum band intensity. If a test is measured using a stationary detector, which is looking at a particular point on the strip, than a positioning error of the strip can give severe erroneous results and lead to a completely wrong diagnostic.

4.1.2 Off-Axis Versus Confocal Measurements

Besides the positioning of the membrane in the horizontal orientation, its positioning in the vertical orientation is also important. The distance between the detector and the strip is crucial to guarantee reading
reproducibility. For any optical measurement system, the sensitivity of detection changes with distance of the sample to the detector. Usually this is expressed as numerical aperture. This factor is particularly critical for off axis illumination, in which changes in distance may result in a total loss of signal. The use of confocal detection still guarantees a signal on the detection point even with position shifts.

4.1.3 Accurate, Sufficient and Homogeneous Illumination of the Sample

An improper strip illumination causes incorrect readings by the detector, independent of it being stationary, scanning, CCD/CMOS or any other system. A system that illuminates a larger area, such as an entire lateral flow strip at a time, must provide homogeneous illumination over the entire strip. The intensity of illumination is also very important to achieve the best sensitivity.

4.1.4 Numerical Aperture, Field of View and Sensitivity

The closer the detector is to the illuminated sample, the more photons it can capture. That is why it is important to position the detector close to the sample. But if the capture device is positioned close to the sample, it can not view the entire strip and the images at the edges get distorted due to the wide angles. A confocal scanner can be positioned very close to the sample and since it scans, it does not need to view the entire strip at a time.

4.2 Calibration Curve

To obtain the concentration of the analyte, a concentration curve has to used. This curve relates the values obtained in the measurement, to a know value of analyte in the sample. The shape of the concentration curve dictates the degree of accuracy of the results. If the analyte measurement falls in the linear range of the curve, its concentration can be easily determined. In the other hand if the analyte measurement fall in the asymptotic sections of it, no determination of the analyte concentration may be possible at all.

4.3 Image Acquisition and Quantification

As stated previously, there are equipments which perform rapid test quantification to a certain level. These equipments are usually dedicated, and can present high costs to the final user, loosing the test’s final main characteristic; performing an assay at a low cost with high confidence levels in delivering a qualitative result.
The use of a table scanner as a possible reader, presents several advantages and also some downsides, which are worth exploration.

The main advantages about the possibility of using a table scanner are its low cost, facility to be replaced, possibility of reading several devices simultaneously, presenting confocal measurements, possibility of treating positioning errors, enabling sufficient lighting, closeness to the test device and homogenous lighting in all test’s readings. The major downside of using it as an equipment is the loss of mobility, restringing the test quantification to more lab type environments.

4.3.1 Quantification Procedure and Results

To perform the test’s quantification, the test devices are run according to their protocols detailed in listings 3.1, 3.2 and 3.3. Right after the test’s last minute of run, the devices are carefully placed on the scanning area. The lid is closed and the image is acquired at 1200 DPI (dots per inch). The image is than manually cropped, taking attention to repeat the same region of interest in each test. Such region correspond in it’s majority to the reaction matrix as can be seen in figure 4.2. Any misalignment, is digitally corrected, rotating the image, until the test and control lines are vertically aligned.

From the figure 4.2 several aspects can be noticed. Amongst them are:

- The inferior region of the test presents a shaded area. This shade is due to the cassette’s geometry which blocks lighting to such region. Although this could present as a challenge to solve, it was identified that it was reproducible to every image acquisition;

- Small variations during the manual adjustment could possibly cause great levels of variation to the test’s final quantification. An extra cropping was than performed to each test device focussing more in the interior area of the test and making the cropped images standard in size as can be seen in figure 4.5.

- Each image is formed by three distinct matrixes, each one corresponuding to one of the color components, red, green and blue. To perform further analysis with the images they have to be transformed into grayscale data.
Once the image is lastly treated, it is converted to gray scale using the standard percentual weightings for three-color components as is detailed by equation 4.1, in which, Xrgb is the final pixel value after each pixel is corrected by its respective index. R, G and B are the values of the pixels in the matrixes that correspond to the red, green and blue components respectively [29].

\[ X_{rgb} = 0.2990 \times R + 0.5870 \times G + 0.1140 \times B; \]  

(4.1)

Since the colored images are now converted into a single matrix, it is necessary to further simplify the information and extract features from the images. It was noticed that if the result of each column is summed or averaged, the regions where the test and control line appear, present lower absolute values than areas which are mainly white. Adding or averaging each column and plotting them against their respective position reveals the test strip intensity profile, as can be seen in figure 4.4.

Figure 4.4 shows the intensity profile of the test result presented in figure 4.3. The image and the profile shows that the areas closer to the image’s borders could interfere negatively with the final result outcome. Cropping more extensively which leaves a more central zone could refine the test’s result.

![Figure 4.3: Test image after initial cropping and conversion to gray scale](image)

The new cropped image can be seen in figure 4.5 and its respective intensity profile can be seen in figure 4.6. For what can be inferred from the image, removing the edge areas, which present several interferents, improves the signal noise ratio, improving the possibility of a more accurate reading and quantification process.

4.4 QUANTIFYING THE HIV TEST

4.4.1 Calibration Curve

To perform an accurate determination of a certain analyte, a calibration curve is needed. This calibration curve is composed of a set of samples of known concentration. The tests are run with the set of samples and the intensity of the band is read. Each intensity is than related to a
known sample concentration and a graph is plotted relating the known concentrations with the intensity read in the test.

To create the set of known concentrations, hyperimmune goat serum was used. This serum was used to create a dilution series. The hyperimmune serum was diluted with regular non-infected human serum. The samples were applied to the devices and the images were acquired. Each one of the serums was also dosed using an ELISA kit, and the results can be seen in the third column of table 4.1. The sample dilutions can be seen in the second column of the same table. Following what was previously discussed, images were converted to gray scale and cropped isolating the central region which is of the most interest. Figure 4.7 demonstrates the intensity profiles of the samples with known concentrations. The cropped regions of each one of the tests from dilution 1:1 to dilution 1:1024 can be seen in figure 4.8.

As can be noticed, several issues previously discussed can be perceived even from such a small set of samples. The largest problem which must be addressed is the positioning error. During the assembly procedures, the strips can be slightly placed further up or down in
the cassette. This positioning error can cause large errors during the quantification phases.

Further analyzing the intensity profiles generated by the calibration samples, several aspects can be noticed. The first is that in samples that have too great of an analyte concentration, the test line may end up presenting such a high nanoparticles concentration that its intensity can be greater than the control line, although in real patients samples this would be very rare. The final point that can be inferred is that the intensity profile correlates with the image and the test result. Even if a sample presents a higher concentration than another, its strip may give a more intense test and control lines. Although this result is not expected, it can happen, but the intensity profile is representative of the result obtained in the test strip.

4.4.2 Method for Quantifying the Test

An initial exploration of the test quantification performance was done by analyzing the calibration curve results. The best method for quantifying the test is determining the least sensitivity needed for the test. In the HIV's case, it is the last control concentration which was still visible, for it, a dilution of $10^2$.

To calculate the test line intensity of each device, a safe area around the test region must be considered when summing the pixels together. Although such slack on each side of the test line increases the background, such artifice is needed to guarantee that minor assembly
errors become less relevant in quantifying the test. The table 4.1 shows the values obtained calculating the area of the intensity curve for the test zone in each device of the calibration curve and the average pixel value also of the test region, which is also shown in figure 4.9 and 4.10.

From table 4.1 and figures 4.9 and 4.10, can be determined that the intensity of the test image behaves linearly, with an $R^2 = 0.937$ if the sample concentration is transformed in $\log_2$. Also, as expected, the use of the average pixel value or the absolute value of all pixels summed in the regions give the same result. Another important point that can be perceived from the test results, perhaps the most important point is that in the test’s visual cutoff, which is the dilution of 1 to 1024, was also the limit of image measuring sensibility. Beyond that point, all results are considered negative, both for visual and image based interpretations.

### 4.4.3 Quantifying Real Patient Samples

A set of known characterized patient samples is used to test the image analysis methodology. The most important point to verify, is if the samples fall inside the calibration curve in concordance to the results obtained from other methodologies. Table 4.2 shows the results of ELISA measurements and their respective intensity values. These values were plotted in the calibration curve which corroborated the calibration initially performed with the HIV hyperimmune goat serum.
### 4.5 Quantifying the HCV Test

#### 4.5.1 Calibration Curve

Similar to the HIV test, an accurate determination of the HCV antibody titer requires a calibration curve. The set of samples was run with the test devices and each intensity was plotted being related to a known concentration obtained by ELISA. This can be seen in the third column.

**Table 4.2: Samples for Calibration**

<table>
<thead>
<tr>
<th>Classification</th>
<th>ELISA result</th>
<th>LFIA quantification result</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+ 1</td>
<td>234 (+)</td>
<td>3782522</td>
</tr>
<tr>
<td>HIV+ 2</td>
<td>423 (+)</td>
<td>3792304</td>
</tr>
<tr>
<td>HIV+ 3</td>
<td>19 (+)</td>
<td>4145502</td>
</tr>
<tr>
<td>HIV+ 4</td>
<td>177 (+)</td>
<td>3912824</td>
</tr>
<tr>
<td>HIV+ 5</td>
<td>209 (+)</td>
<td>3992733</td>
</tr>
<tr>
<td>HIV- 1</td>
<td>0,3 (-)</td>
<td>4759265</td>
</tr>
<tr>
<td>HIV- 2</td>
<td>0,2 (-)</td>
<td>4785938</td>
</tr>
<tr>
<td>HIV- 3</td>
<td>0,8 (-)</td>
<td>4729622</td>
</tr>
</tbody>
</table>

The table shows the samples used for calibration, with ELISA results and LFIA quantification results for both HIV+ and HIV-.
of table 4.3. To obtain the intensity from the bands, similarly as it was performed for the HIV test, in the HCV, the images were cropped, align and a second cropping operation was performed. To create the set of samples a hyperimmune goat serum was diluted with non infected human serum.

The sample dilutions can be seen in the second column of table 4.3. Figure 4.12 demonstrates the intensity profiles of the samples with known concentrations. The cropped regions of each one of the tests from dilution 1:1 to dilution 1:1024 can be seen in figure 4.13. Similarly to the HIV, the HCV test calibration curve presented an $R^2 = 0.9505$.

<table>
<thead>
<tr>
<th>Point</th>
<th>Dilution</th>
<th>Value in ELISA</th>
<th>AUC intensity</th>
<th>Average AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1</td>
<td>212</td>
<td>3976157</td>
<td>195.8602</td>
</tr>
<tr>
<td>2</td>
<td>1:2</td>
<td>103</td>
<td>4139082</td>
<td>203.8856</td>
</tr>
<tr>
<td>3</td>
<td>1:4</td>
<td>61</td>
<td>4128544</td>
<td>203.3665</td>
</tr>
<tr>
<td>4</td>
<td>1:8</td>
<td>48</td>
<td>4241688</td>
<td>208.9399</td>
</tr>
<tr>
<td>5</td>
<td>1:16</td>
<td>24</td>
<td>4290254</td>
<td>211.3322</td>
</tr>
<tr>
<td>6</td>
<td>1:32</td>
<td>12</td>
<td>4289663</td>
<td>211.3030</td>
</tr>
<tr>
<td>7</td>
<td>1:64</td>
<td>6,5</td>
<td>4573175</td>
<td>225.2685</td>
</tr>
<tr>
<td>8</td>
<td>1:128</td>
<td>3,1</td>
<td>4517639</td>
<td>222.5328</td>
</tr>
<tr>
<td>9</td>
<td>1:256</td>
<td>1,65</td>
<td>4543965</td>
<td>223.8296</td>
</tr>
<tr>
<td>10</td>
<td>1:512</td>
<td>0,82</td>
<td>4769651</td>
<td>234.9466</td>
</tr>
<tr>
<td>11</td>
<td>1:1024</td>
<td>0,48</td>
<td>4768396</td>
<td>234.8848</td>
</tr>
</tbody>
</table>

4.5.2 Method for Quantifying the Test

In the same manner as the HIV, the least sensitivity needed for the test is than determined. In the HCV’s case, it is the last control concentration which was still visible, for it, a dilution of 1 to 256.

To calculate the test line intensity of each device, a safe area around the test region was also considered when summing the pixels together. Table 4.3 shows the values obtained calculating the area of the intensity curve for the test zone in each device of the calibration curve and the average pixel value also of the test region, which is also shown in figure 4.14 and 4.15.

4.5.3 Quantifying Real Patient Samples

A set of known characterized patient samples is used to test the image analysis methodology. As before for the HIV, the most important
point to verify, is if the samples fall inside the calibration curve in concordance to the results obtained from other methodologies. Table 4.4 shows the results of ELISA measurements and their respective intensity values. These values were plotted in the calibration curve which corroborated the calibration initially performed with the HCV hyperimune goat serum that was used to create a dilution series; which can be seen in figure 4.16.

<table>
<thead>
<tr>
<th>Classification</th>
<th>ELISA result</th>
<th>LFIA quantification result</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV+ 1</td>
<td>12 (+)</td>
<td>4582522</td>
</tr>
<tr>
<td>HCV+ 2</td>
<td>16 (+)</td>
<td>4492304</td>
</tr>
<tr>
<td>HCV+ 3</td>
<td>19 (+)</td>
<td>4345502</td>
</tr>
<tr>
<td>HCV+ 4</td>
<td>8 (+)</td>
<td>4672824</td>
</tr>
<tr>
<td>HCV+ 5</td>
<td>4 (+)</td>
<td>4542733</td>
</tr>
<tr>
<td>HCV- 1</td>
<td>0,5 (-)</td>
<td>4809265</td>
</tr>
<tr>
<td>HCV- 2</td>
<td>0,7 (-)</td>
<td>4835938</td>
</tr>
<tr>
<td>HCV- 3</td>
<td>0,1 (-)</td>
<td>4729622</td>
</tr>
</tbody>
</table>

4.6 QUANTIFYING THE FOB TEST

4.6.1 Calibration Curve

Unlike the HIV and HCV, which are antibody detection tests, the FOB, as previously stated is a test which detects antigen in the sample. Also to determine the haemoglobin concentration a calibration curve is required. Several human haemoglobin solutions were prepared to build a calibration curve. The solutions where 500ng/mL, 400ng/mL, 300ng/mL, 200ng/mL, 100ng/mL, 50ng/mL, 40ng/mL, 25ng/mL and 0ng/mL. These solutions besides the haemoglobin, presented the same constitution of the transport buffer previously stated in subsection 3.5.4.5. The intensity results for the FOB quantification can be seen in table 4.5. Figure 4.18 demonstrates the intensity profiles of the samples with known concentrations. The cropped regions of each one of the test’s solutions can be seen in figure 4.17.

4.6.2 Method for Quantifying the Test

To quantify the FOB test results, the test area was cropped and the pixels added and averaged. This procedure revealed the test line intensity
of each device. Also a safe area around the test zone was considered to reduce the impact of assembly errors during quantification. Table 4.5 shows the values obtained calculating the area of the intensity curve for the test zone in each device of the calibration curve and the average pixel value also of the test region, which is also shown in figure 4.19 and 4.20.

4.6.3 Quantifying Real Patient Samples

Unlike the HIV and HCV tests, which provided more consistent results, the FOB intensity difference was very small when comparing standards up to ten times more concentrated. Besides that, there are no easily available methodologies to quantify the amount of haemoglobin in real patient stool samples. Because of this real patient characterization loses purpose since there is no reference value to compare the obtained result to.
Figure 4.8: Cropped regions of an HIV test battery with samples presented in table 4.1. The first image corresponds to the first sample in the table and the last image corresponds to the last sample.
Figure 4.9: Calibration curve obtained adding the pixel values of the test zone area for the HIV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the $\log_2$ of the ELISA result with the intensity value obtained from the image.
Figure 4.10: Calibration curve obtained averaging the pixel values of the test zone area for the HIV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the $\log_2$ of the ELISA result with the intensity value obtained from the image.
Figure 4.11: Intensity against ELISA values obtained with patient sample, plotted together with calibration curve previously determined.

Figure 4.12: Intensity profiles of the HCV calibration test battery with samples of known concentration.
Figure 4.13: Cropped regions of an HCV test battery with samples presented in table 4.3. The first image corresponds to the first sample in the table and the last image corresponds to the last sample.
Figure 4.14: Calibration curve obtained adding the pixel values of the test zone area for the HCV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the log2 of the ELISA result with the intensity value obtained from the image.
Figure 4.15: Calibration curve obtained averaging the pixel values of the test zone area for the HCV tests. The superior graph relates the ELISA result with the intensity value obtained from the image. The inferior graph relates the $\log_2$ of the ELISA result with the intensity value obtained from the image.
Figure 4.16: Intensity against ELISA values obtained with patient sample, plotted together with calibration curve previously determined.
Figure 4.17: Cropped regions of an FOB test battery with samples presented in table 4.5. The first image corresponds to the first sample in the table and the last image corresponds to the last sample.
Figure 4.18: Intensity profiles of the FOB calibration test battery with samples of known concentration

Figure 4.19: Calibration curve obtained adding the pixel values of the test zone area for the FOB tests. The superior graph relates the standard solution concentration with the intensity value obtained from the image. The inferior graph relates the log$_2$ of the solution concentration with the intensity value obtained from the image.
Figure 4.20: Calibration curve obtained averaging the pixel values of the test zone area for the FOB tests. The superior graph relates the standard solution concentration with the intensity value obtained from the image. The inferior graph relates the log2 of the standard solution concentration with the intensity value obtained from the image.
Part IV

DISCUSSION AND CONCLUSION
CONCLUSION

In this chapter the results obtained in the previous chapters and sections are further discusses and a conclusion to this work is presented.

5.1 TESTING PLATFORM’S EVOLUTION

The diagnostic market has reached what can be called as point of no return. Most of the technological trends and developments are leading to the arising of a universal test platform which incorporates the advantages of the rapid tests platform with the resources of the ELISA and CLIA tests. Several efforts have been made to convert the most popular test platform today, which is the lateral flow test assay, into a quantifiable reading method.

The main challenge regarding the quantification of lateral flow tests, is that these tests have not been designed from their beginning to allow reproducible quantifiable results. Several aspects which are embedded in the manufacturing process pose as a challenge to allow reproducible systematic quantifiable results.

Although the LFIA may never reach the level of assertiveness needed to be used confidently as a true quantifiable test, the holly grail of diagnostics which is a low cost, easy access and utilization with reproducible quantifiable results is relentless being searched.

5.2 HIV TESTING

HIV is a serious world healthcare problem. Although the infection rate is controlled in several populations, there are many countries, specially in the Subsaharan Africa. In Brazil there are several populations in which the infection rate is growing and in others the rate has stabilized. Either way, the necessity of mass testing several populations is evident. Since Brazil is not technology nor production wise self sufficient, the development of a testing device alone is of the most importance strategically for the country to become independent of high valued imported products.

Besides the test development, the exploration of a possible quantification methodology adds tactile scientifically value to a well established platform in order to reduce one of its main shortcomings.
5.2.1 HIV Test Device

The HIV test device developed presented good results when tested against a series of known characterized samples. The main challenge in developing such tests, once their core protein is selected, is adjusting all test parameters which are required. The protein concentration adjustments are tricky and can be the turning point in having a high quality test or a test with undesirable features.

From tables 3.2 and 3.3, can be seen that little adjustments can reduce significantly the false positive or negative rate of incidence. Although membrane blocking is an extra step during manufacturing, it some times is of the essence to avoid false positive results. After the test’s fine tuning, a larger batch of samples is used to put the developed test to the proof. Table 3.4 and figure 3.6 show the overall performance of the developed device. One false positive result was obtained in a sample pool of over 100 negative samples. No false negative results were present. Since the HIV rapid test is intended to be a screening method. If a positive result is obtained, other methodologies must be used to confirm the result. Having a high sensitivity test with an acceptable specificity is acceptable. Although ideal, having perfectly sensible and specific tests is still a task to be accomplished.

5.2.2 HIV Test Device Quantification

Quantification in lateral flow is a challenge to be accomplished. From the acquired results, semi-quantification is achievable. Even a more reliable qualitative result can also be obtained. Figures 4.9 and 4.10 show that the visual detection limit is essentially the same as the one obtained by the quantification method. This demonstrates that an assured qualitative result, which removes the human flaw component is easily obtained. The calibration curve if plotted in terms of \( \log_2 \) also presents a linear behavior across most of it’s length. Using real characterized samples, the intensity result obtained by the calibration corroborates the result previously obtained for those samples. This indicates a reasonable level of assertiveness for the method. These results are shown in table 4.2 and figure 4.11.

5.3 HCV TESTING

HCV is also a serious world healthcare problem. Maybe even more concernable than the HIV, the HCV is known as the silent killer. The infection rate has presented significant growth in several populations and may even be considered an epidemic in some countries. The proportion of infected individuals has grown significantly in recent years. The necessity of mass testing most of the populations is evident in recent times. Similarly as the HIV, for the HCV Brazil is not technology
nor production wise self sufficient in developing testing solutions to
attend the government and private sector needs. The development of
a testing device alone is of the most importance strategically so the
country may become independent of imported products.

Similarly as it was for the HIV, besides the test development, the
even further exploration of a possible quantification methodology
adds even more scientifically value to a well stablished platform.

5.3.1 HCV Test Device

Traditionally, developing a high quality HCV rapid test has proven
to be extremely difficult. Obtaining acceptable sensitivity, specially
in low titer samples has always been a challenge to this kind of test.
The new platform, which fuses the flow-through with the lateral flow,
has shown acceptable levels of sensitivity and specificity. The results
seen in table 3.7 and figure 3.7 show the efficiency of the developed
test device. All known positive samples presented positive results
in the test. Only one negative sample gave a false positive result
out of 100 samples. As before the test presented higher sensitiveness
than specificity. For screening type of tests, this is exactly the needed
performance.

5.3.2 HCV Test Device Quantification

The HIV rapid test quantification presented acceptable results which
indicate that such quantification method can be applied to several
cases. The HCV quantification presented similar results as the HIV.
The results can be seen in table 4.3 and 4.4. This comes to corroborate
the applicability of the method, since two different tests, which use
the sample platform, but non the less two different tests, have given
very similar results.

5.4 FOB TESTING

The research of haemoglobin in fecal matter is a good indicative of a
possible tumor in the lower intestines. Although colonoscopy is the
gold standard to diagnose colorectal cancer, this is an invasive exam
which brings several risk and discomfort to patients. The screening of
patients for intestinal bleeding is the best strategy to ensure patient
compliance. Those individuals which present blood in their stool
sample are indicated to perform the colonoscopy.

Although there are other alternatives to detect haemoglobin in fecal
matter, such as guaiac based and heme porphyrin tests, those exams
present a higher level of lab preparation and usually require that
the patient engage in dietary restrictions prior the exam. The FOB
immunological detection exam is a single step easily performed exam in which dietary restriction is unnecessary.

The development and manufacturing of such a test device by itself already constitutes a great deal of scientific work and unification of several technological trends. Adding the exploration of quantification to such a needed test, concludes the exploration of the lateral flow technology proposed in this work.

5.4.1 FOB Test Device

Different from the HIV and HCV tests, the FOB is an antigen detection test. With a higher demand even than HIV and HCV, this test format today is probably most lab’s preferred choice in testing for fecal occult blood.

The test device presented excellent results in haemoglobin’s research in prepared controls and real patient samples. The test calibration was carefully performed to ensure an optimal device’s response. The device’s performance was presented in tables 3.11, 3.12 and 3.13. Figure 3.10 show the final test’s ROC curve.

5.4.2 FOB Test Device Quantification

The final test device quantification did not present as good results as were seen for the HIV and HCV. The main difficulty encountered, was the low test line intensity and background created by the test run. Figures 4.18 and 4.17 show a different situation than what as previously encountered. The strongest the test line appears, the “cleaner” the test’s overall background is. The test line captures the colloidal gold which migrates through the strip. An excess colloidal gold which migrates trough the strips does not flow entirely during the 10 minutes run time. Because of this situation the quantification results can not be acknowledged as being trustworthy. There are several strategies which can be used to surpass that problem. The easiest two of them are the use of faster flowing membranes, which would clear the background inside the 10 minute window, or increasing the window of reading to 15 to 20 minutes. Both of them though require that the test is completely redone and re standardized.

5.5 Conclusion

The most important aspect of this work, which was perceived during the overall development of all three tests and the quantification method, is that if all aspects of the test are not known in detail, attempting to perform rapid test quantification will most likely be unsuccessful. There are so many control points which vary from test to test, from manufacturer to manufacturer that a reader which would
attend all of the test in the market or at least its majority is task to be accomplished.

Another point which must be attended is that rapid tests are an excellent platform for field and in lab testing, but they have several downsides which are at least tricky to be solved. Committing further time and effort in developing quantification methods for rapid tests will most likely not achieve the level of confidence that can be obtained using other and newer test platforms.

Since most of the manufacturing process and overall technology for rapid test development is available, the use releasing new products in the market is relatively easy. Besides further exploring the technology, this work intended to supply material for other researchers to catalyze a new era in diagnostics in Brazil. An era in which manufacturing from beginning to end of high end products is possible and accessible.
This dissertation was typeset with \LaTeX{} using Classic Thesis available for \LaTeX{} via CTAN as “classicthesis”.

BIBLIOGRAPHY


"Mama always said life was like a box of chocolates. You never know what you’re gonna get."

Forrest Gump’s mother.