Behavior Exploration for Model Selection of Image Based Result Quantification of Newly Developed HIV, HCV and FOB Lateral Flow Device Tests

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Abstract—**Lateral Flow immunoassays, have been developed in the 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 colloidal gold conjugates, 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. Although simple, such solution can broaden the applications of lateral flow technology.**

Lateral flow immunnoassays technology is well stablished, trustworthy and is around since the 1970's [6]. The regular growth of its use with the development of new tests for new parameters comes to demonstrate the large applicability of such technology [20]. For a number of years, lateral flow technology has been used in commercial tests for the detection of HIV, HCV, Dengue, Chagas amongst other infectious diseases. A large deficiency of such tests is the lack of quantification methodology for the assays. Most tests make use of colloidal gold labels [11] which are colored particles and allow visual test interpretation and simplifies the utilization of the test device.

Although very popular and with several advantages, regularly seen rapid tests, present one main shortcoming which is the lack of quantification methodologies and easy to use equipments to perform final result quantification of the test [17]. This deficiency causes the technology to suffer in several types of assays in which quantification is of the essence and also final result visual interpretation may be flawed because it has the human component which is always subject to error. The combination of both competences in a final test methodology is rarely seen.

The work presented here includes the development of three different lateral flow test devices and a quantification method for the final result which resulted in a clear linear model and could be used to calibrate a quantitative test response or to obtain a qualitative ensured result. The tests developed englobe 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. After the image acquisition, the results were quantified and several features were extracted from the images. The features were analyzed and was perceived that central tendency data such as average and median presented a behavior which coincided with the predicted test response from the biological end, which is the largest the amount of analyte in the sample, the strongest the final result becomes presenting a linear behavior.

The obtained results finally showed that the use of image analysis techniques associated with feature selection analysis allows the obtaining of more reliable qualitative results and also possibly semi-quantitative results. The results also showed that true quantification requires extra work, specially on the biological/chemical end of the assay and that test quantification does not compensate for flawed and inconsistent test devices. Perhaps the most important issue addressed in this work is that a simple low cost solution which uses simple image analysis and modeling techniques can address a major concern of a well know and stablished technology which seeks to broaden its uses and applications.

I. RAPID-TESTS (LATERAL FLOW IMMUNOASSAYS)

The lateral flow imunochromatographic 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 [3]. 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 [14].

The first and main application derived from the imunochromatographic test platform was the human pregnancy test [13]. 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

were nitrocellulose membrane manufacturing, antibodies and antigens production and purification [9].

A. Architecture of a Lateral Flow Imunoassay

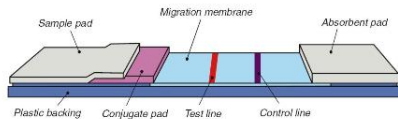The figure 1 shows the basic configuration of a Lateral Flow Test.

Fig. 1.   Lateral Flow Test Components

The test is basically formed by a sample pad, backing sheet, conjugate pad, test line, control line, nitrocelulose membrane and absorbent pad. As can be seen in figure 1. The parts overlap amongst them and are mounted on a plastic backing material which gives support and holds the components in place. When a test is run, the sample is usually added to the sample pad. The sample migrates thought 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 microbeeds. The sample mobilizes the conjugate which interacts with the analyte in sample. The analyte along with the conjugate migrates into the nitrocellulose membrane. As it migrates, they keep on interacting, until the test line is reached. The test line is composed either of antibodies or antigens, depending on the test configuration, which capture the complex analite-conjugate [1].

B. HIV Diagnostic

As of december 2003, there were approximately 40 million people living with HIV/AIDS worldwide [15]. HIV diagnostic methods have improved deeply the care of patients. Diagnostic methods are based on the detection of 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.

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 tests, a further overview will be performed in this item. 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 [10].

C. HCV 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 are 1,2,3,4,5 and 6 [21].

Diagnostic tests for HCV detection can be divided into immunological assays, which detect anti-HCV antibodies and molecular tests which detect viral particles. The most important screening test is the detection of anti-HCV IgG in serum because once a person has sero-converted, they remain positive for HCV antibodies.

HCV assays include first, second and third generation assays. In recent years HCV's RNA detection by the use of reverse transcriptase polymerase chain reaction (RT-PCR) has become an essential tool in diagnosis of HCV infection. This kind of assays is specially important in immunocompromised patients which do not develop an immune response. Rapid HCV 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 [16].

D. Fecal Haemoglobin Detection - FOB

Colorectal cancer is among the most common cancers both in male and female individuals. Several studies have demonstrated that diet plays a large roll in colon cancer development and prognosis. The use of early-detection screening tests for colorectal cancer has the potential to remove precancerous polyps and improve survival prospects for patients with diagnosed cancer. Stool testing is a non-invasive, widely accepted simple technique for colorectal cancer screening. The traditional guaiac-based test, reacts to the presence of haem group. In recent years immunochemical test, which reacts to the presence of globin has gained more acceptance due to it's higher sensitivity and specificity characteristics [22].

Rapid tests which detect haemoglobin in faecal matter are the preferable test coniguration for this test. In this type of assays, the specimen added to a transport solution. This solution in than added and absorbed into a pad and immediately combined with a signal reagent. The specimen-signal reagent migrates though the reaction matrix (nitrocelullose membrane) and if the test is positive, a line (in the test region) appears [7].

II.   HIV, HCV AND HAEMOGLOBIN DETECTION (FOB) RAPID TEST DEVELOPMENT

HIV and HCV are tests which detect antibodies in the sample. The Haemoglobin detection test, detects an antigen which is the Haemoglobin. Essentially both test configurations are different.

The antibody detection tests (HIV and HCV) uses antigens impregnated in the nitrocelulose membrane and the colloidal gold is conjugated to Protein A. In this configuration, once the sample is added, the conjugate links to most of antibodies in the sample. The specific antibodies against the impregnated antigen are captured in the test line and the remainder of antibodies are captured in the control line. which is also protein A. The antigen detection test (Haemoglobin detection) uses a

pair of antibodies. One antibody is conjugated to the colloidal gold and another is impregnated to the test line. The control line is constituted by a third antibody which is specific to Fc portion of the antibody conjugated to the gold nanoparticles.

A. HIV Test Development

Perhaps one of the most crucial parts of rapid test development is the protein selection. The antigen used came from Meridian Lifescience. The antigen is constituted of an envelope portion conjugated to gp36, giving immunogenic response to HIV-2 virus. The protein also contains the C terminal portion of gp120 and part of gp41. This gives reactivity to HIV-1, HIV-2 and subtype O.

The test line is constituted of an HIV antigen which comes in 1,0 mg/mL , SDS 0,1% pH 8.0 and 50mM Tris buffer. To be impregnated in the membrane we diluted the protein solution in a 1,0 mg/mL albumin solution, SDS 0,1%, sucrose 5% and azide 0,1%. The final antigen concentration was 0,125 mg/mL. The control line is constituted of a Protein A 1,0 mg/mL, azide 0,1% solution. Both the control and test lines are dispensed using an AUTOKUN contact dispenser which dispenses $30\mu L$ in a 30 cm nitrocelulose sheet.

The 15 nm colloidal gold particle solution is formulated adding equal volumes of 1% gold chloride and 4% sodium citrate solutions to a boiling water beaker. After adding the sodium citrate solution, the boiling solution changes from gray to a strong red and finally to a burgundy color. The colloidal gold nano particles were conjugated to a protein A solution. The gold was titrated to determine the least amount of protein A which stabilizes the solution. During titration was determined that 1mL, of 1 mg/mL protein A solution stabilizes 120 mL of gold solution. The protein A was added to the gold solution and stirred vigorously for about 60 seconds. The solutions was left overnight resting under mild agitation. After overnight stabilization, 5% of a 20% Albumin solution was added and 10% of a concentrated phosphate buffer solution was also added. The albumin protect and stabilizes the gold and the phosphate buffer brings the gold to a final 7,4 pH. After final pH adjustment, the colloidal gold is diluted 1:1 with a 6% tween-20 and 10% albumin. This conjugate is liquid stable.

The test procedure is very basic and is the same for the HCV test. Two drops of the liquid conjugate is added to the test device in the area marked with an "R". After the reagent reaches the area marked with a "T" 5 μL of serum is added to the window marked with an "S". If the sample presents antibodies against HIV, they are captured in the test line and the test comes out positive. If not, only the control line appears. A positive HIV test result may be seen in figure 2

Fig. 2.   HIV test with positive result

B. HCV Test Development

In the same manner as the HIV, perhaps the most crucial part of rapid test development is the protein selection. The anti-

gen used also came from Meridian Lifescience. The antigen is constituted of recombinant nucleocapside/NS3/NS4/NS5 . It contains the imunodominant regions of nucleocapsides NS3, NS4 and NS5. It also contains GST fusion agent. The final molecular weight is of 70,8 KDa.de 70,8kDa.

The test line is constituted of an HCV antigen which comes in 1,0 mg/mL , SDS 0,1% pH 8.0 and 50mM Tris buffer. To be impregnated in the membrane we diluted the protein solution in a 1,0 mg/mL albumin, SDS 0,1%, sucrose 5% and azide 0,1% solution. The final antigen concentration was 0,200 mg/mL. The control line is constituted of a Protein A 1,0 mg/mL and azide 0,1% solution. Both the control and test lines are dispensed using an AUTOKUN contact dispenser which dispenses $30\mu L$ in a 30 cm nitrocelulose sheet. The conjugate used was the one prepared for the HIV.

Fig. 3.   HCV test with positive result

C. Haemoglobin Detection Test Development - FOB

Unlike the HIV and HCV, the Haemoglobin detection test performs a sandwich on the antigen in the sample. One antibody is conjugated to the gold nanoparticle and another is impregnated in the nitrocelulose membrane. A crucial portion of this test is the selection of the antibody pair. The antibodies came from Chemux Biotechnology and have presented specificity to human haemoglobin and high binding constant to different haemoglobin portions.

The test line is constituted of an anti-human haemoglobin antibody. The antibody comes in 4,5 mg/mL phosphate buffer, pH 7,4 and azide 0,1%. To be impregnated in the membrane we diluted the antibody solution in a 1,0 mg/mL albumin solution, sucrose 5% and azide 0,1% solution. The final antigen concentration was 0,150 mg/mL. The control line is constituted of a Protein A1,0 mg/mL, azide 0,1% solution. Both the control and test lines are dispensed using an AUTOKUN contact dispenser which dispenses $30\mu L$ in a 30 cm nitrocelulose sheet.

In the same manner as the HIV and HCV tests, the 15 nm colloidal gold particle solution was formulated adding equal volumes of 1% gold chloride and 4% sodium citrate solutions to a boiling water beaker. After adding the sodium citrate solution, the boiling solution changes from gray to a strong red and finally to a burgundy color. The colloidal gold nano particles were conjugated to the other anti-human haemoglobin antibody which pairs up with the antibody on the nitrocelulose membrane. The gold was titrated to determine the least amount of antibody which stabilizes the solution. During titration was determined that 1mL, of 1 mg/mL antibody solution stabilizes 64 mL of gold solution. The antibodies were added to the gold solution and stirred vigorously for about 60 seconds. The solutions was left overnight resting under mild agitation. After overnight stabilization, 5% of a 20% albumin solution was added and 10% of a concentrated phosphate buffer solution was also added. The albumin protects and stabilizes the gold

and the phosphate buffer brings the gold to a final 7,4 pH. After final pH adjustment, the colloidal gold receives a 2% sucrose and was freeze dried into fiberglass membranes over a 48 hours period. The fiber glass sheets were dipped into the solution, the excess was removed from the sheet and they were placed into stainless steel trays for the freeze-drying procedure. A final solution must be prepared for the test, the transport solution. A 100 mM phosphate and 0,5% Triton-X100 solution is prepared. This solution is placed in transport plastic tubes with 2mL each.

The test procedure is very basic but differs from the HIV and HCV procedures. A straw with gravels is inserted into 6 different spot in recently collected feces. This straw is added to the buffer solution and the solution stirred. Finally two drops from this solution is added to the test's sample pad and the result is seen with 5 to 10 minutes. A positive FOB test result may be seen in figure 4

Fig. 4.   FOB test with positive result

III.   Image Acquisition and Quantification

The images were acquired with the use of a HP scanner. All test in a battery were simultaneously ran and the image for all test devices were also simultaneously acquired. The HIV and HCV tests were ran twice with a battery of serial dilutions of a known high control. The haemoglobin test was ran against a series solutions with different known haemoglobin concentrations. The images from each device were identically analyzed. The region comprehending the test line was isolated giving a manually adjusted margin (repeated for each test device). This margin is of the essence because during test assembly there is an inherent variation of the line position. The image with the line was converted to a real number representation, so it could be manipulated.

After each test was scanned and had the respective test areas isolated, the images were quantified. Since the image may be seen as a matrix, each pixel has it's intensity along with the X and Y axis which give it's position. This can be seen in figure 5. To process the image each pixel in a line was added together. Since each pixel in white presented the highest possible value, 256. Each pixel that was black presented the value of 1, the values in the pixels of the test line region varied according to the antibody or antigen concentration in the sample. Each image was constituted of three matrixes (R,G,B), the values or the three matrixes were averaged to compose a final matrix.

The values of each line corresponding to the test line region were linearly added. The final result of the lines had several characteristics extracted. Amongst them are average, maximum, minimum, median, variance, standard deviation and skewness. A first analysis of the characteristic extracted from the samples has demonstrated that the best features to be used were the central tendency ones, because they represented the
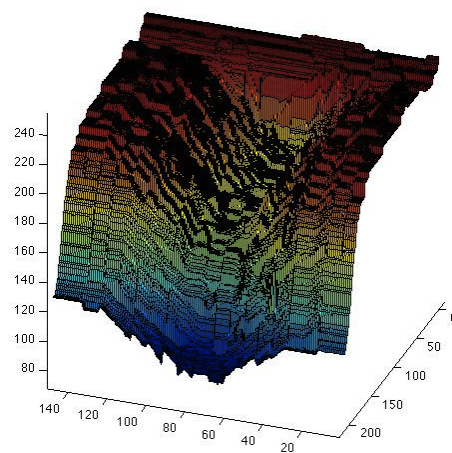

Fig. 5.   3D image of a Test Line Region

assay's expected behavior. Initial trials can be seen in figures 6 and 7. They were performed with an HCV test battery. This initial test quantification has demonstrated that average and median present linear responses with the increase of the analyte in the sample. Maximum and variance do not present a linear response. The minimum and skewness have also presented linear responses, but the central tendency measurements have been selected due to their robustness.
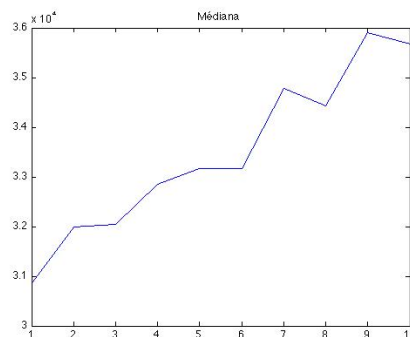

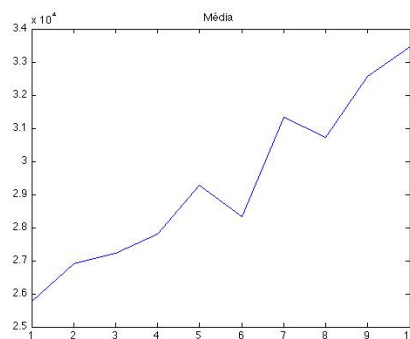Fig. 6.   Test line quantification response for the HCV - median

Fig. 7.   Test line quantification response for the HCV - average

Central tendency measurements such as median and average, presented a linear behavior when they are related

to the antigen or antibody concentrations. The largest the antibody/antigen concentration in the sample, the lowest the final value of the overall intensity in the graph. Point one in the graph present a high concentration sample and point 10 present a lower concentration sample. The most important aspect that can be extracted from this response is that the antibody/antigen concentration presents a linear response with the overall image intensity. This behavior can be seen in figure 6 and figure7.

IV. Test Performance Evaluation and Final Quantification Assessment

A. HIV Test

A lot made up of about 400 units was produced for testing. From these, 200 units were tested with 100 know negative samples to ensure that the test does not present false positive results. All tests were performed twice. The tests presented 100% specificity since there were no false positive results. From this lot 100 units were tested with 50 known positive characterized samples. The test presented 100% sensitivity since all tests presented positive results. All tests were also performed twice.

A high titer positive control was used in a dilution series. A total of 12 samples obtained in dilutions from 1 to 4096. This dilution series was then used to run a total of 24 tests; twice for each sample. The final test results were than scanned and the test line area isolated and quantified. One behavior that was expected was that the higher the antibody concentration in the sample, the lower the average of the overall value of the test. This was demonstrated as can be seen in figure 8
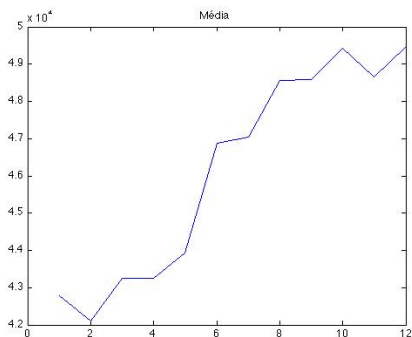

Fig. 8.   Test line quantification response for the HIV - average

In the graph the first point is the concentrated control and the last point is the control diluted by a factor of 4096. The behavior seen in figure 8 just demonstrates a linear tendency when related to a dilution series.

This behavior was expected because the test is a sequential assay, which means that the largest the amount of antibody in the sample, the strongest the signal becomes, until a certain limit when all the antigen sites have been occupied.

B. HCV Test

In the same manner as the HIV, a lot made up of about 400 units was produced for testing. From these, 200 units were tested with 100 know negative samples to ensure that the test does not present false positive results. All tests were performed

twice. The tests presented 100% specificity since there were no false positive results. From this lot 100 units were tested with 50 known positive characterized samples. The test presented 100% sensitivity since all tests presented positive results. All tests were also performed twice.

A high titer positive control was used in a dilution series. A total of 10 samples was then formed in dilutions from 1 to 1024. This dilution series was then used to run a total of 20 tests, twice for each sample. The final test results were than scanned and the test line area isolated and quantified. One behavior that was expected and happened for the HIV, was that the higher the antibody concentration in the sample, the lower the average of the overall value of the test. This was demonstrated as can be seen in figure 9
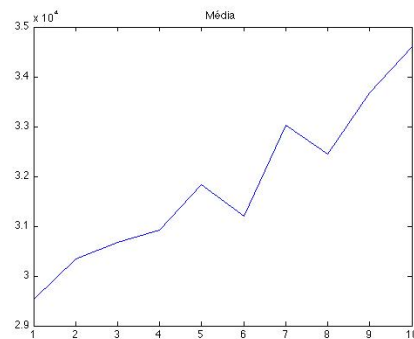

Fig. 9.   Test line quantification response for the HCV - average

In the graph the first point is the concentrated control and the last point is the control diluted by a factor of 2048. The behavior seen in figure 8 just demonstrates a linear tendency when related to a dilution series. This behavior was also expected because the test is a sequential assay, as was the HIV. This means that the largest the amount of antibody in the sample, the strongest the signal becomes, until a certain limit when all antigen sites have been occupied.

C. FOB Test

With the FOB test, a lot made up of 200 tests was produced for testing. From these, 50 units were tested with 25 know negative stool samples to ensure that the test does not present false positive results. All tests were performed twice. The tests presented 100% specificity since there were no false positive results. From this lot 50 units were tested with 25 known positive characterized samples. The test presented 100% sensitivity since all tests presented positive results. All tests were also performed twice.

Solutions of know haemoglobin concentrations were used. A total of 10 solutions with different concentrations were applied to the test. The concentrations varied from 40ng/mL to 4000ng/mL. Each sample was run in duplicate. The final test results were than scanned and the test line area isolated and quantified. One behavior that was expected was that the higher the antigen concentration in the sample, the lower average of the overall value of the test. This was not demonstrated as can be seen in figure 10.

In the graph the first point is very diluted haemoglobin solution. The last point is a solution 400 times more concen-
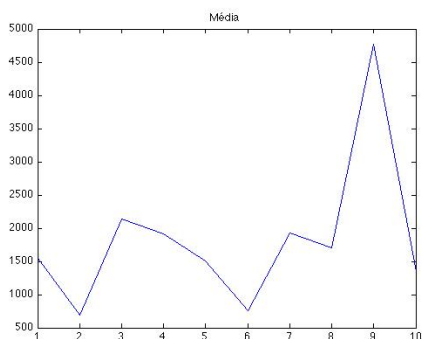
Fig. 10. Test line quantification response for the FOB - average

trated. The behavior seen in figure 10 demonstrates a variation which was not expected for the assay results.

A linear behavior was also expected for this case, like the one seen on the HIV and HCV. Analyzing the possible causes for it, the most plausible one is the low amount of antibody used in the test line. Such low amount guarantees the high specificity of the test, but interferes directly with the final signal intensity. The use of higher amounts increases the test's sensitivity but causes the test to become to sensitive. If very small amounts of antibody are in the test, even if the amount of haemoglobin is very large, the intensity of the test line becomes constant, because all antibodies binding sites are occupied.

V. CONCLUSION

The main weakness of visually based rapid test is the lack of quantification methodologies and also the possibility of human error during visual interpretation. The use of a simple methodology for the test's quantification can considerably increase the robustness of rapid-test's interpretation even if the aimed result is just qualitative.

One of the main points identified during the tests and quantification methodology developments is that there is a great necessity of adjusting the final test behavior to optimize the quantification methodology. Spending great deals of time and efforts on quantification methodologies and equipments will not solve problems related to the chemical and biological aspects of the test. Most of the effort must be focussed in developing highly reproducible robust assays. With those assets at hand, test quantification is greatly simplified.

Although rapid test quantification is the next step on the lateral flow technology, the greatest requirement which would bring the greatest value for the tests immediately is a solid qualitative result without the possibility of human error. This procedure may be easily implemented by creating a certain level of intensity in the image in which below it, the test is negative and above it, the test is positive.

The intent of this work was to explore the behavior of the rapid tests and see if the expected results could be achieved. As predicted the best feature to be obtained from the tests is the average of their overall intensity value despite several other central tendency measurements could have been used. There is still much room to work on both the biological-chemical and

the electronics ends of the overall testing methodology but a linear response is expected for the test methodology.

VI. ACKNOWLEDGMENT

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