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Experimental Validation Form

I. **Protocol Title: Data Analysis Workflow-** Verification of RGB Calculation and Bar Plot Generation

II. **Brief Description of Protocol:** RGB calculation and bar plot verification protocol aims to ensure the efficiency and accuracy of RGB calculation and generation of bar plots for the test strips. Steps involved in this are Data Preparation: Collect the test strip images and extract RGB values for each image. Then calculate the Average pixel values for each component R, G, and B. Verify by comparing with the values or known reference values. Verification of bar plot generation by visually inspecting them to see if they are expected patterns and trends and if they correspond with the correct RGB components. Show different test cases for comparison.

III. **Hypothesis:** Hypothesize that the implemented code for RGB calculation and bar plot generation accurately represents the color intensity of the test strips and produces visually informative bar plots

IV. **Types of Data:** The type of data in this protocol is image data. Specifically, test strip images are used for analysis and processing.

V. **Controls:** The protocol does not explicitly mention specific control groups or experimental controls. However, control images or reference images can be used for comparison and to establish a baseline for analyzing changes in the test strip images.

VI. **Number of Samples:** The number of samples is not explicitly mentioned in the protocol. The number of samples will depend on the specific experimental setup or the number of test strip images available for analysis. The protocol can be applied to any number of test strip images as per the experimental requirements.

VII. **Experimental Justification:**
The experimental justification for this protocol lies in the analysis and interpretation of RGB values, bar graph plots, and pixel changes in test strip images. By calculating the average pixel values for each color channel, visualizing the RGB values through bar graph plots, and quantifying pixel changes, the protocol aims to provide insights into color variations, color intensity, and potential substance presence in the tested region of the test strip images. These analyses can be valuable for applications such as chemical analysis, quality control, and image-based diagnostics.

VIII. **Experimental Limitations:**

- The sample size was small and limited and due to this statistical power and reliability of the results are restricted to the performed experiments.
- Variability in the test strips can cause uncertainty in results and this could influence the measurements or outcomes.

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- The performance of the test strip can be influenced by reagent sensitivity and stability. Factors such as variations in reagent quality or storage conditions might also affect the test results.
- Image Analysis Accuracy and pixel calculator can be influenced by the lighting condition, resolution, and algorithm limitation (linear scale).
- Some other limitations are the lack of a control group, assumptions, equipment limitations, and human error.

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I. **Protocol Title: Data Analysis Workflow-** Verification of RGB Calculation and Bar Plot Generation

II. **Hypothesis:** Hypothesize that the implemented code for RGB calculation and bar plot generation accurately represents the color intensity of the test strips and produces visually informative bar plots
    A. **Null Hypothesis(H0):** There is no significant color change is observed in the spiked drink and no difference in average RGB values across test strips.
    B. **Alternative Hypothesis(Ha):** There is a significant difference observed in the average RGB values across different test strips.

IX. **Materials:**
- Test strip images saturated with modified Scotts Reagent
- Alcohol Solution containing Benedryl (substitute for ketamine)
- Smartphone Camera or imaging device for capturing test strip images
- Anaconda: Python programming environment
- Jupyter Notebook: Data Analysis and Visualization
- Required Python Libraries: os, cv2, numpy, matplotlib.pyplot

X. **Procedure:**
1. **RGB Calculation:**
    a. Star by setting the working directory to the folder that has all the reaction test strip images.
    b. Import the required/necessary libraries such as OpenCV, NumPy, and Matplotlib.
    c. Load the images using the cv2.imread() function and store them in separate variables.
    d. Now compute the average pixel values for Red, Green, and Blue color channels in each image using cv2.mean() and store them in separate variables.
    e. Convert the average pixel values to a list and Using the round() function round them to two decimal places.
    f. Print the average pixel values for each color channel for each image.
2. **Bar Graph Plotting:**
    a. The previously obtained RGB values will now be subtracted from 255 to enhance the visual representation for better interpretation. (Detailed explanation is given on pages 2 and 3)
    b. Set and define the labels, values, and colors for the bar graph plot.
    c. Use a for loop to loop through the clause list and plot a bar graph for each image using plt.bar() function.
    d. Add a legend to the bar graph chart using plt.legend.
    e. Set labels for the x-axis and y-axis using plt.xlabel and plt.ylabel.
    f. Set the chart title using plt.title.
    g. Show the plot using plt.show() function.

XI. **Guidelines for Interpreting Results and Making the Data-Driven Decisions**

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1. **RGB Values Interpretation:**
    a. The calculated RGB values represent the average pixel intensity for each individual color channel (i.e. Red, Green, Blue) in the focused region of the test strip images.
    b. The higher the RGB value, the higher the intensity of that color channel in the image.
    c. Concept and Interpretation:
    d. Compare the RGB values between the non-tested ad tested regions of the test strip to identify any significant differences.
    e. A change in the RGB values suggests a color change and presence of the drug in the tested region compared to the non-tested region.
2. **Bar Graph Interpretation:**
    a. The bar graph visually represents the RGB values for each image.
    b. Compare the heights of the bars to identify any significant changes or differences between the images.
    c. The RGB graphs are also available in a normalized form for an overall representation.
    d. A higher bar indicates a higher normalized RGb value representing a higher intensity of the merged RGB channels. This helps identify patterns or trends in the normalized RGB values like the variation between different test strip images or other regions of the same test strip.
3. **Explanation of RGB Values and Subtraction for Visual Representation**
    a. RGB values represent the intensity of red, green, and blue colors in an image. Each color channel has a value from 0 to 255, where 0 is the absence of that color and 255 represents the maximum intensity of that color.
    b. In relation to image analysis, darker color tends to have lower RGB values because they absorb more light and brighter colors have higher RGB values as they reflect more light, while brighter colors have a much higher RGB value as they reflect more light. So as the color gets darker the RGB value decreases.
    c. When plotting Bar graphs to represent the RGB values it is challenging to interpret results directly as color intensity is inversely related to RGB values. To resolve this issue and improve visual representation a simple transformation can be applied.
    d. We subtracted the RGB values from 255 to invert the color intensity and now the darker colors get the higher values and the brighter colors get a lower value. This is better for a more intuitive interpretation of the bar graph, as higher values indicate a more intense color and lower values indicate a less intense color.
    e. This makes it easier to compare and draw conclusions based on the relative intensities of different colors in the test strip images.

Note: This transformation does not alter the relative difference between the colors; it simply enhances the visual representation for better interpretation.

Results and Data Analysis:

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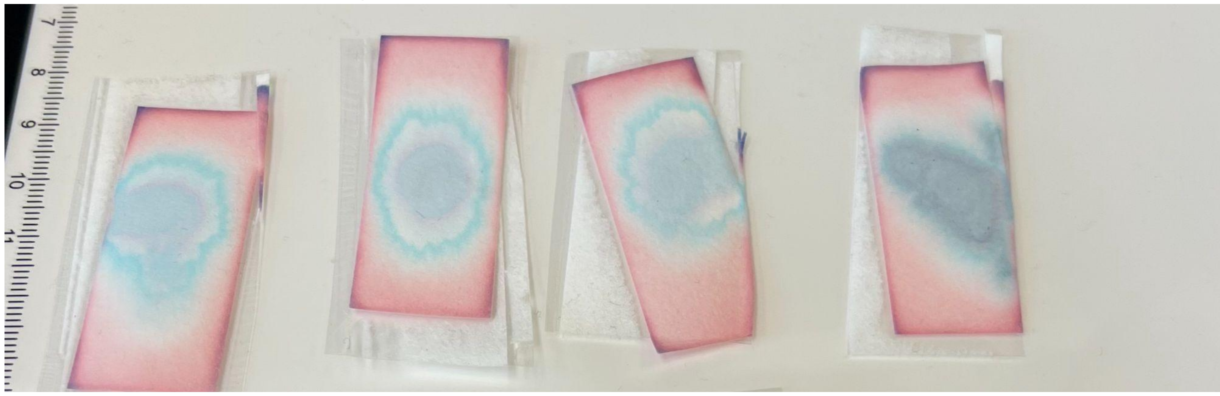
- **Test Case 1:** Captured image on smartphone



- Below used Photoshop to edit and get the focused area (region of interest where the reaction occurred) which makes it easier to view it.

Figure 1a

Figure 1b

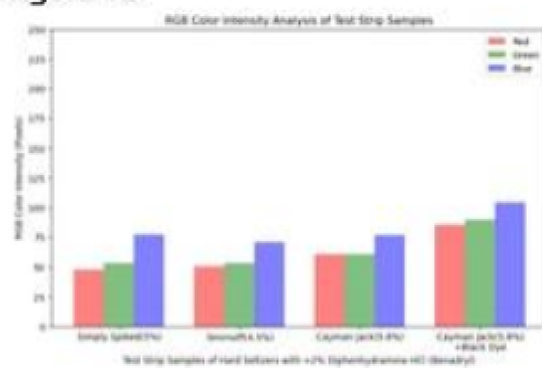


Figure 1a. Single-sided laminated test strips testing different types of seltzer solutions, all at different alcohol concentrations (4.5% - 5.8%) all containing a 2% Benadryl concentration **Figure 1b.** RGB Color intensity via pixels via Python

Calculated RGB output:

```
Below we print the average pixel values of R, G, B component in Simply Spiked(5%) tested strip
[207.01, 201.64, 177.72]
Below we print the average pixel values of R, G, B component in Smirnoff(4.5%) tested strip
[204.42, 201.83, 184.44]
Below we print the average pixel values of R, G, B component in Cayman Jack(5.8%) tested strip
[194.21, 194.35, 178.62]
Below we print the average pixel values of R, G, B component in Cayman Jack(5.8%)+Black Dye tested strip
[169.87, 165.69, 150.87]
```

Subtract the RGB Output from 255 for better graphical interpretation and visualization:

```
# Define the data
labels = ['Simply Spiked(5%)', 'Smirnoff(4.5%)', 'Cayman Jack(5.8%)', 'Cayman Jack(5.8%)\n+Black Dye']
red_means = [255-207.01,255-204.42, 255-194.21, 255-169.87]
green_means = [255-201.64,255-201.83, 255-194.35, 255-165.69]
blue_means = [255-177.72,255-184.44, 255-178.62, 255-150.87]
```

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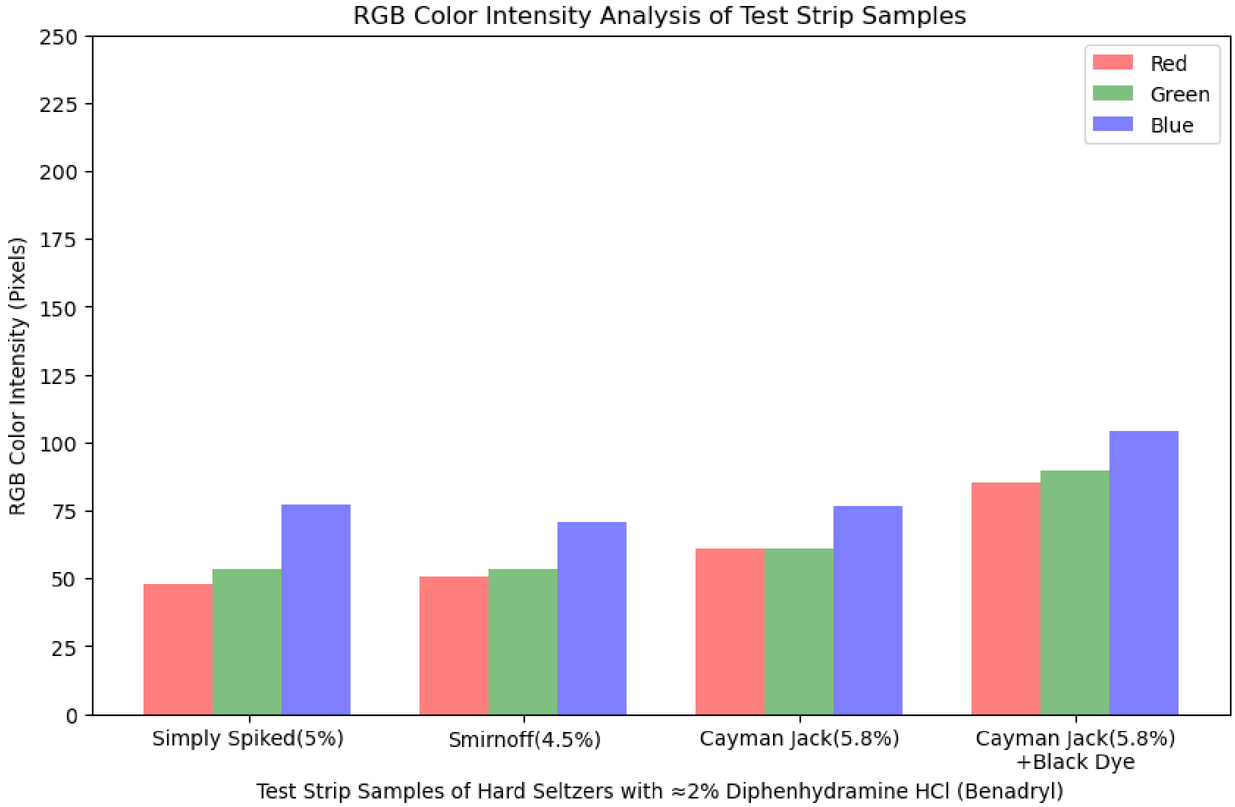
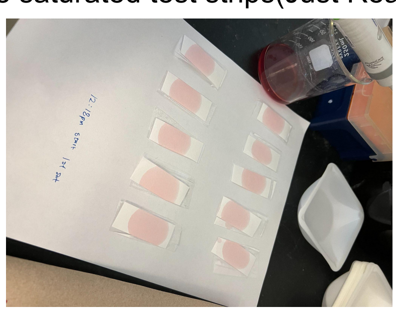
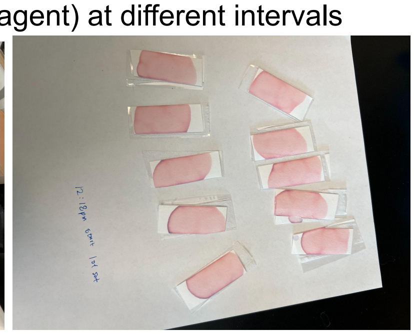
A better and clear view of the Graph above:



We tested our test strip in 3 different alcohol solutions to check if the color change occurs and every test gave a positive result as benedryl was indeed present. Now to double confirm we added black dye to Cayman Jack to see if the test strip is actually working and not giving a false positive that was also a successful test which gave a dark blue color after the test strip came in contact with the solution.

- **Test Case 2:**
  Captured images of the saturated test strips(Just Reagent) at different intervals

Below are the focused images of the test strip from each time interval

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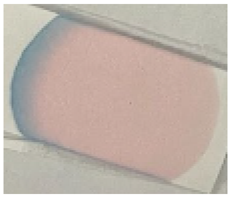
  

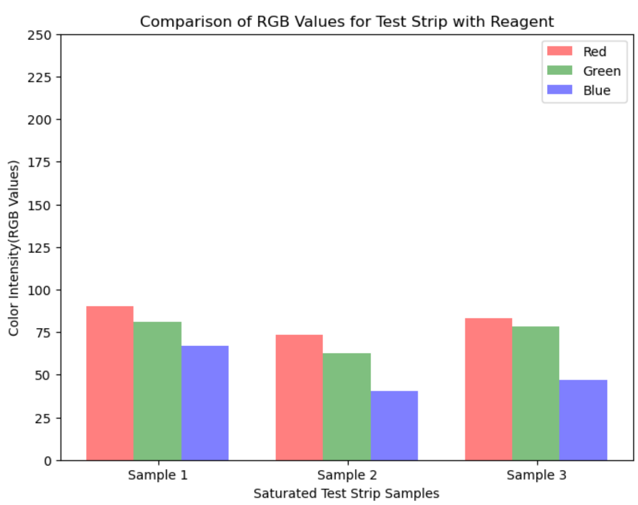
Calculated RGB output:

```
Below we print the average pixel values of R, G, B component in test strip image-1
[164.59, 174.12, 188.31]
Below we print the average pixel values of R, G, B component in test strip image-2
[181.4, 193.14, 214.79]
Below we print the average pixel values of R, G, B component in test strip image-3
[171.67, 176.61, 208.08]
```

Subtract the RGB Output from 255 for better graphical interpretation and visualization:

```python
# Define the data
labels = ['Sample 1', 'Sample 2', 'Sample 3']
red_means = [255-164.59,255-181.4, 255-171.67]
green_means = [255-174.12,255-192.14, 255-176.61]
blue_means = [255-188.31,255-214.79, 255-208.08]
```

Generated Bar Graph that corresponds to the above data:



- **<u>Test Case 3</u>**

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Figure 2a

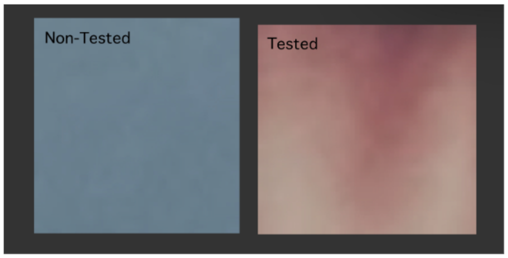

Figure 2b.

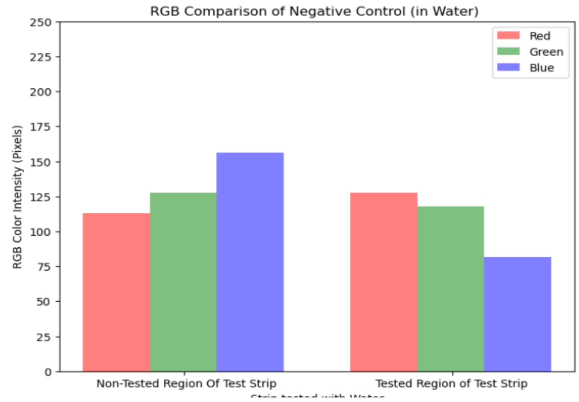

Figure 2a. Color change in the presence of diphenhydramine. Non-tested is darker blue due to a loss of coordinated water molecules. This pink test strip was rehydrated.
Figure 2b. RGB color intensity between the non-tested test strip and the tested strip.

- **Test Case 3:**

```
Below we print the average pixel values of R, G, B component in Non-test region of test strip:
[137.54, 141.49, 139.7]
Below we print the average pixel values of R, G, B component in Tested region of test strip (Benedryl+Kambucha:
[127.57, 115.77, 86.97]
```

```
# Define the data
labels = [' Non-Tested Region Of Test Strip', ' Tested Region of Test Strip ']
red_means = [255-137.54,255-127.57]
green_means = [255-141.49,255-115.77]
blue_means = [255-139.7,255-86.97]
```

<- Captured image from smartphone and the below is the focused area of interest

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Figure 3a

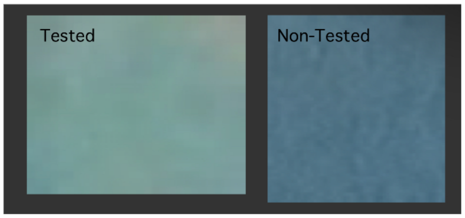


Figure 3b

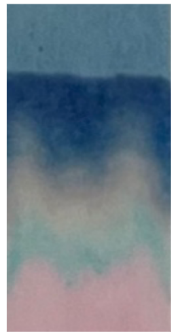


Figure 3c

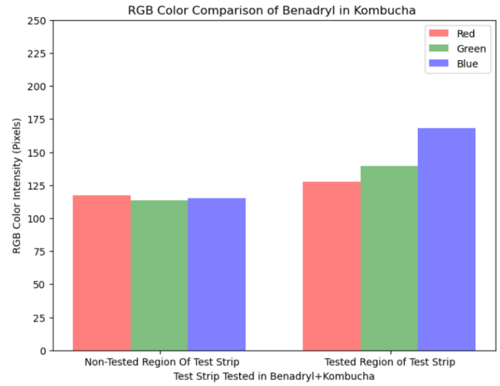
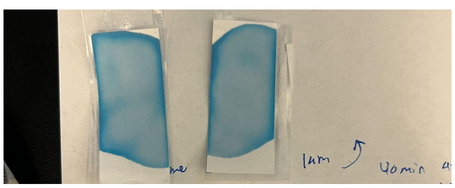


Figure 3a. The left test strip displays a blue /teal color change caused by 40% alcohol containing 2% benadryl concentration. Right test strip demonstrates a darker blue color on a non-tested region with loss of coordinated water molecules.**Figure 3b** RGB color intensity between the non-tested strip and the tested strip 5 distinct colors from top to bottom: non tested loss of water molecules (blue), (darker blue) how high the solution reached, (white) separation between positive result and non testing region, (bright blue/teal) positive result, (pink) rehydration of reagent
Figure 3c. RGB color intensity between the non-tested strip and the tested strip.

- **Test Case 4:**


Reagent with Loss of Water After 30min

```
Below we print the average pixel values of R, G, B component after 30 minutes (Test Srtip 1):
[149.37, 147.99, 120.6]
Below we print the average pixel values of R, G, B component after 30 mintes(Test Strip 2):
[134.31, 132.38, 104.91]
```

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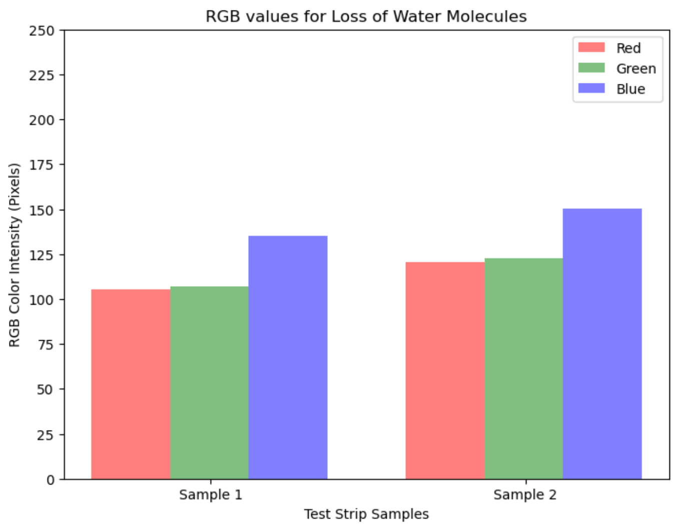
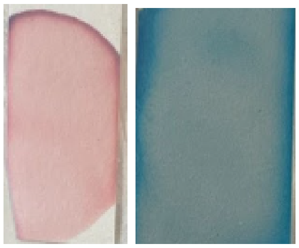
```
# Define the data
labels = ['Sample 1', 'Sample 2']
red_means = [255-149.37,255-134.31]
green_means = [255-147.99,255-132.38]
blue_means = [255-120,255-104.91]
```



The test strip loose water molecules and there it turns from pink to blue before any testing is done. This is reflected in the bar graph above.

Test Case 5:



```
Below we print the average pixel values of R, G, B component in Test Strip with Reagent:
[171.67, 176.61, 208.08]
Below we print the average pixel values of R, G, B component in Test Strip with Reagent and Loss of Water Moelcules:
[133.18, 129.92, 100.62]
```

```
labels = ['Test Strip with Reagent', 'Test Strip with Reagent \n and Loss of Water Molecule']
red_means = [255-171.67,255-133.18]
green_means = [255-176.61,255-129.92]
blue_means = [255-208.08,255-100.62]
```

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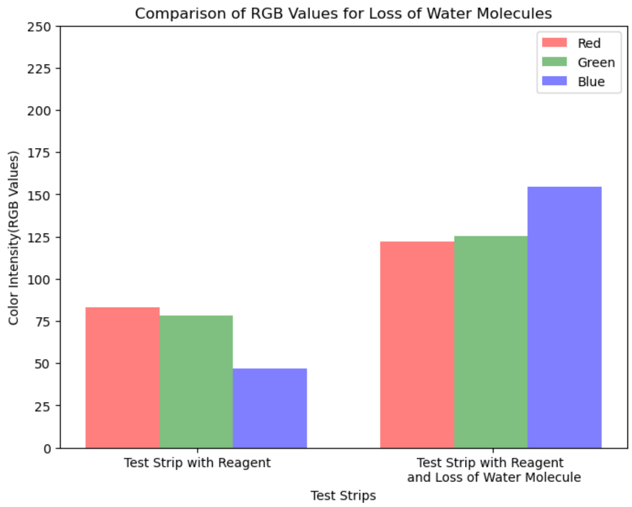
Below is a comparison of the Test Strip with Just the Reagent and after 30mins of Reagent with loss of water:



A clear difference in the test strip with reagent and with loss of water molecule.

- **Test Case 6:**

```
Below we print the average pixel values of R, G, B component in Test Strip After 19 days(1):
[181.47, 171.83, 155.92]
Below we print the average pixel values of R, G, B component in Test Strip After 19 days(2):
[187.68, 177.91, 164.95]
Below we print the average pixel values of R, G, B component in Test Strip After 19 days(3):
[171.46, 163.25, 149.09]
```

```
# Define the data
labels = ['Sample 1', 'Sample 2', 'Sample 3']
red_means = [255-181.47,255-171.83, 255-155.92]
green_means = [255-187.68,255-177.91, 255-164.95]
blue_means = [255-171.46,255-163.25, 255-149.09]
```

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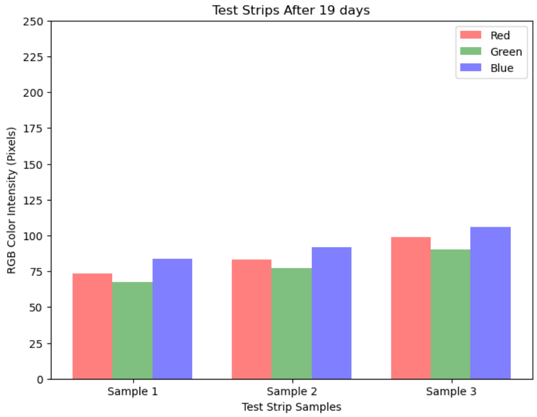

The above shows the Test strip after 19 days

Future Work (if more time was there):

- More testing with more research leads to improvements that can address the above-mentioned limitation and enhance the robustness of the findings/results.

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References

1. Prasannahariveeresh, “Implementing Lane Detection in OpenCV Python: A Step-by-Step Beginner’s Guide - Prasannahariveeresh.” *Prasannahariveeresh - Read My Tech Blogs Here*, 18 Jan. 2023, https://jrprasanna.com/2023/01/18/implementing-lane-detection-in-opencv-python-a-step-by-step-beginners-guide/
2. “Matplotlib Pie Charts.” *Vegibit*, vegibit.com/matplotlib-pie-charts/. Accessed 19 May 2023. https://vegibit.com/matplotlib-pie-charts/