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# Task 1: Data Handling and Statistical Analysis

**Objective:** Assess candidates' ability to handle complex data and apply statistical methods effectively.
**Background:** CpG methylation is an epigenetic marker that varies across tissue types. However, the methylation status of a single CpG site is unreliable as a biomarker due to errors introduced by bisulfite sequencing, sampling techniques, and biological variability.

**Definition**: Phased Methylation Pattern (PMP) is a unique set of coordinates that includes the DNA strand ('f' for forward (+) or 'r' for reverse (-)), the relative positions of three CpG sites on the same strand (e.g., x:y:z), and their methylation status (e.g., '000' for all unmethylated or '111' for all methylated). It represents a combined epigenetic signature across these CpG sites.

**Hypothesis**: Phased methylation patterns (PMPs) can act as reliable biomarkers to differentiate tissue types, providing higher specificity compared to individual CpG sites.

- Strand: Indicates the DNA strand ('f' or 'r').
- CpG Coordinates: Relative positions of three CpG sites (x:y:z).
- Methylation Status: Eight possible patterns ('000' to '111').
- Sample ID: Unique identifier for each sample.
- Replicate: Indicates technical replicates.
- Tissue: Tissue type (Tissue #1 or Tissue #2).

## Sub-tasks:

### 1. Coverage Analysis

**a.** Calculate the median and coefficient of variation (CV) for single CpG coverage in each tissue.

coverage analysis ensures the integrity and reliability of methylation data by addressing sequencing biases, variability, and depth requirements. It is foundational to validating hypotheses like the use of PMPs as tissue-specific biomarkers.

***Code-***

```
import pandas as pd


df = pd.read_csv('PupilBioTest.csv')

# Columns for single CpG coverage
cpg_columns = ['`000', '`001', '`010', '`011', '`100', '`101', '`110', '`111']

# dictionary to store metrics for each cpg column
results = []

# loop through each CpG column
for cpg in cpg_columns:
    # grouped by tissue
    grouped = df.groupby('Tissue')[cpg].agg(
        Median='median',
        Mean='mean',
        StdDev='std'
    ).reset_index()


    grouped['CV'] = grouped['StdDev'] / grouped['Mean']

    # add a column to indicate which CpG column this analysis belongs to
    grouped['CpG_Column'] = cpg

    # append the result for this CpG column to the results list
    results.append(grouped)

# merge all results
final_results = pd.concat(results, ignore_index=True)

final_results.to_csv('single_cpg_coverage_statistics.csv', index=False)
print(final_results)
```

***Result***

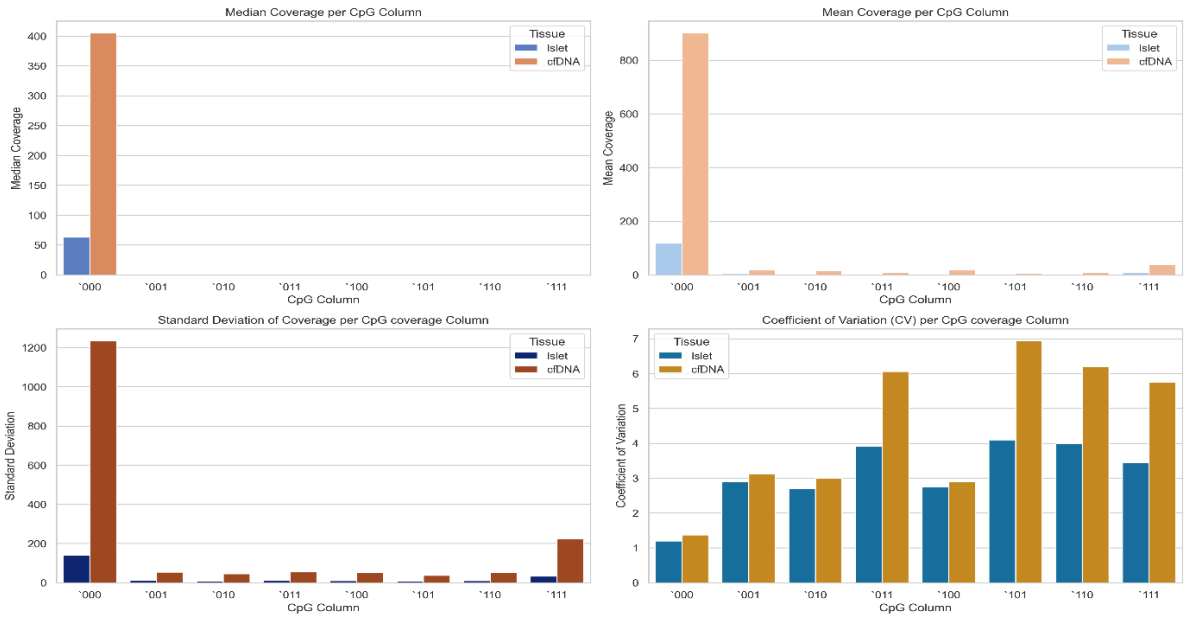
| Tissue | Median | Mean | StdDev | CV | CpG_Column |
|---|---|---|---|---|---|
| Islet | 63.0 | 118.13349410305797 | 141.62176745066844 | 1.1988282284033658 | `000 |
| cfDNA | 405.0 | 901.1693246807491 | 1233.919834983006 | 1.3692430503225774 | `000 |
| Islet | 0.0 | 4.402681459592095 | 12.755096521792158 | 2.8971200026299213 | `001 |
| cfDNA | 0.0 | 17.330327752352744 | 54.140656430768956 | 3.1240411147688127 | `001 |
| Islet | 0.0 | 3.589680219519274 | 9.730252986511097 | 2.7106183257221064 | `010 |
| cfDNA | 0.0 | 15.344915994826101 | 46.0824017094427 | 3.0031055057571163 | `010 |
| Islet | 0.0 | 3.0605838896071345 | 11.95712251151349 | 3.9068109036698684 | `011 |
| cfDNA | 0.0 | 9.161572279736319 | 55.53826962485141 | 6.062089336749725 | `011 |
| Islet | 0.0 | 3.6812419197382655 | 10.099163136752452 | 2.743411967195706 | `100 |
| cfDNA | 0.0 | 17.711453279553336 | 51.28316726196754 | 2.89548048104954 | `100 |
| Islet | 0.0 | 2.356704572438722 | 9.66620599857126 | 4.101577309101859 | `101 |
| cfDNA | 0.0 | 5.381630005577446 | 37.341554225842735 | 6.938707080780817 | `101 |
| Islet | 0.0 | 2.6243500171499434 | 10.50249161329049 | 4.001940116469771 | `110 |
| cfDNA | 0.0 | 8.538781699803389 | 53.00663873823008 | 6.207751948905146 | `110 |
| Islet | 0.0 | 9.510754069813462 | 32.84428030086858 | 3.4533834078534604 | `111 |
| cfDNA | 0.0 | 38.87023304349745 | 223.4799323434775 | 5.7493849366273135 | `111 |

**b.** Generate plots summarizing the coverage statistics.

***Code-***

```
import pandas as pd
import seaborn as sns
import matplotlib.pyplot as plt

#plot
results = pd.read_csv('single_cpg_coverage_statistics.csv')


sns.set(style="whitegrid")
plt.figure(figsize=(16, 10))

# 1. Median plot
plt.subplot(2, 2, 1)
sns.barplot(data=results, x='CpG_Column', y='Median', hue='Tissue', palette='muted')
plt.title('Median Coverage per CpG Column')
plt.xlabel('CpG Column')
plt.ylabel('Median Coverage')

# 2. Mean plot
plt.subplot(2, 2, 2)
sns.barplot(data=results, x='CpG_Column', y='Mean', hue='Tissue', palette='pastel')
plt.title('Mean Coverage per CpG Column')
plt.xlabel('CpG Column')
plt.ylabel('Mean Coverage')

# 3. Standard Deviation plot
plt.subplot(2, 2, 3)
sns.barplot(data=results, x='CpG_Column', y='StdDev', hue='Tissue', palette='dark')
plt.title('Standard Deviation of Coverage per CpG coverage Column')
plt.xlabel('CpG Column')
plt.ylabel('Standard Deviation')

# 4. CV plot
plt.subplot(2, 2, 4)
sns.barplot(data=results, x='CpG_Column', y='CV', hue='Tissue', palette='colorblind')
plt.title('Coefficient of Variation (CV) per CpG coverage Column')
plt.xlabel('CpG Column')
plt.ylabel('Coefficient of Variation')

plt.tight_layout()
plt.savefig('coverage_statistics_plots.png', dpi=300)
plt.show()
```

***Result –***



## 2. Biomarker Identification

**a**. Identify PMPs with high specificity for tissue differentiation, minimizing false positives for Tissue #1 while allowing some false negatives. Use statistical or machine learning approaches to assign confidence (e.g., p-values) to each PMP.

***Statistical method-***

***Codes-***

```
import pandas as pd
from scipy.stats import fisher_exact
from statsmodels.stats.multitest import multipletests
chunk_size = 500
file_path = "PupilBioTest.csv"
methylation_cols = ['`000', '`001', '`010', '`011', '`100', '`101', '`110', '`111']

# initialize an empty list to store melted chunks
melted_chunks = []

# process the dataset in chunks
for chunk in pd.read_csv(file_path, chunksize=chunk_size):

    melted_chunk = chunk.melt(
        id_vars=['strand', 'CpG_Coordinates', 'Sample_ID', 'Replicate', 'Tissue'],
        value_vars=methylation_cols,
        var_name='Methylation_Status',
        value_name='Count'
    )
    melted_chunks.append(melted_chunk)

# combine into a single DataFrame
df_melted = pd.concat(melted_chunks, ignore_index=True)

# group data by tissue, strand, coordinates, and methylation status
grouped = df_melted.groupby(['Tissue', 'strand', 'CpG_Coordinates', 'Methylation_Status']).agg(
    Total_Count=('Count', 'sum')
).reset_index()

# Normalize counts by total counts per tissue
grouped['Normalized_Count'] = grouped['Total_Count'] / grouped.groupby('Tissue')['Total_Count'].transform('sum')

#  comparison table
tissue1 = 'cfDNA'
tissue2 = 'islet'

tissue1_data = grouped[grouped['Tissue'] == tissue1]
tissue2_data = grouped[grouped['Tissue'] == tissue2]

comparison = pd.merge(
    tissue1_data[['strand', 'CpG_Coordinates', 'Methylation_Status', 'Total_Count']],
    tissue2_data[['strand', 'CpG_Coordinates', 'Methylation_Status', 'Total_Count']],
    on=['strand', 'CpG_Coordinates', 'Methylation_Status'],
    how='outer',
    suffixes=('_T1', '_T2')
).fillna(0)

# Fisher's Exact Test for each PMP
p_values = []
for _, row in comparison.iterrows():
    contingency_table = [
```

```
            [row['Total_Count_T1'], sum(tissue1_data['Total_Count']) - row['Total_Count_T1']],
            [row['Total_Count_T2'], sum(tissue2_data['Total_Count']) - row['Total_Count_T2']],
        ]
        _, p = fisher_exact(contingency_table)
        p_values.append(p)

    comparison['P_Value'] = p_values

    # adusted p-values for multiple testing with error handling
    if not comparison['P_Value'].isnull().all() and len(comparison['P_Value']) > 0:
        # Remove NaN P-values and apply multiple testing correction
        valid_p_values = comparison['P_Value'].dropna()
        if len(valid_p_values) > 0:
            try:
                comparison['Adjusted_P_Value'] = multipletests(valid_p_values, method='fdr_bh')[1]
            except Exception as e:
                print(f"Error during multiple testing correction: {e}")
                comparison['Adjusted_P_Value'] = None
        else:
            print("Error: No valid P-values found for multiple testing correction.")
            comparison['Adjusted_P_Value'] = None
    else:
        print("Error: No valid P-values found for multiple testing correction.")
        comparison['Adjusted_P_Value'] = None

    # Filter PMPs
    significant_pmps = comparison[comparison['Adjusted_P_Value'] < 0.05]

    significant_pmps.to_csv('statistical_significant_pmps.csv', index=False)
    print("Significant PMPs based on statistical approach saved to 'statistical_significant_pmps.csv'")
```

## ***Result (sample) –***

| 1 | strand | CpG_Coordinates | Methylation_Status | Total_Count_T1 | Total_Count_T2 | P_Value | Adjusted_P_Value |
|---|---|---|---|---|---|---|---|
| 2 | r | 10035:10044:10046 | `000 | 1077.0 | 0.0 | 1.3382072844487038e-36 | 1.7676116401349276e-35 |
| 3 | r | 10035:10044:10053 | `000 | 1086.0 | 0.0 | 5.917675284589969e-37 | 8.023259140671622e-36 |
| 4 | r | 10035:10044:10058 | `000 | 1069.0 | 0.0 | 1.908798556018049e-36 | 2.497462623036202e-35 |
| 5 | r | 10035:10044:10071 | `000 | 1072.0 | 0.0 | 1.9906808891810815e-36 | 2.5918875831200024e-35 |
| 6 | r | 10035:10044:10075 | `000 | 1086.0 | 0.0 | 5.917675284589969e-37 | 8.023259140671622e-36 |
| 7 | r | 10035:10044:10081 | `000 | 1086.0 | 0.0 | 5.917675284589969e-37 | 8.023259140671622e-36 |
| 8 | r | 10035:10044:10088 | `000 | 1047.0 | 0.0 | 1.452516828653845e-35 | 1.810716014159768e-34 |
| 9 | r | 10035:10044:10096 | `000 | 1056.0 | 0.0 | 6.371907791190341e-36 | 8.078305354230393e-35 |
| 10 | r | 10035:10044:10102 | `000 | 1054.0 | 0.0 | 6.226306510017574e-36 | 7.951061266139723e-35 |
| 11 | r | 10035:10044:10109 | `000 | 1005.0 | 0.0 | 3.281449035350466e-34 | 3.921801741714632e-33 |
| 12 | r | 10035:10044:10111 | `000 | 1055.0 | 0.0 | 6.263405682991248e-36 | 7.969487437799825e-35 |
| 13 | r | 10035:10044:10118 | `000 | 955.0 | 0.0 | 1.7386811904872064e-32 | 2.019134815267068e-31 |
| 14 | r | 10035:10044:10121 | `000 | 907.0 | 0.0 | 5.7755991783838745e-31 | 6.59517144230489e-30 |
| 15 | r | 10035:10044:10130 | `000 | 797.0 | 0.0 | 3.550894010064119e-27 | 3.7663965430886996e-26 |
| 16 | r | 10035:10044:10133 | `000 | 764.0 | 0.0 | 3.604335951332221e-26 | 3.7630674200417176e-25 |
| 17 | r | 10035:10044:10140 | `000 | 620.0 | 0.0 | 2.2639107299259707e-21 | 2.165189088302304e-20 |
| 18 | r | 10035:10044:10146 | `000 | 450.0 | 0.0 | 1.6196031197187778e-15 | 1.396467889629982e-14 |

**b.** Calculate the mean variant read fraction (VRF) for each PMP in both tissues.

***Code -***

```
import pandas as pd
file_path = "PupilBioTest.csv"  # Replace with your actual file name
methylation_cols = ['`000', '`001', '`010', '`011', '`100', '`101', '`110', '`111']
df = pd.read_csv(file_path)
df.columns = df.columns.str.strip()
required_columns = ['strand', 'CpG_Coordinates', 'Sample_ID', 'Replicate', 'Tissue'] + methylation_cols
for col in required_columns:
    if col not in df.columns:
        raise ValueError(f"Column '{col}' not found in the dataset. Please check your input file.")
# melt the dataset to create a 'Methylation_Status' column
df_melted = df.melt(
    id_vars=['strand', 'CpG_Coordinates', 'Sample_ID', 'Replicate', 'Tissue'],
    value_vars=methylation_cols,
    var_name='Methylation_Status',
    value_name='Count'
)
# Create PMP
df_melted['PMP'] = (
    df_melted['strand'] + ':' + df_melted['CpG_Coordinates'] + ':' + df_melted['Methylation_Status']
)
# group data by Tissue and PMP to calculate total counts
grouped = df_melted.groupby(['Tissue', 'PMP']).agg(
    Total_Count=('Count', 'sum')
).reset_index()
# Calculate the total counts for each tissue
grouped['Total_Tissue_Count'] = grouped.groupby('Tissue')['Total_Count'].transform('sum')
# Calculate VRF
grouped['VRF'] = grouped['Total_Count'] / grouped['Total_Tissue_Count']
output_file = "vrf_results.csv"
grouped.to_csv(output_file, index=False)
print(f"VRF results saved to {output_file}")
```

**Result –**

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| 1 | Tissue | PMP | Total_Count | Total_Tissue_Coun | VRF | |
| 2 | Islet | f:10035:10044:10046:`000 | 15229 | 558507204 | 2.72673295723505E-05 | |
| 3 | Islet | f:10035:10044:10046:`001 | 145 | 558507204 | 2.59620644033806E-07 | |
| 4 | Islet | f:10035:10044:10046:`010 | 280 | 558507204 | 5.0133641606528E-07 | |
| 5 | Islet | f:10035:10044:10046:`011 | 30 | 558507204 | 5.37146160069943E-08 | |
| 6 | Islet | f:10035:10044:10046:`100 | 101 | 558507204 | 1.80839207223547E-07 | |
| 7 | Islet | f:10035:10044:10046:`101 | 30 | 558507204 | 5.37146160069943E-08 | |
| 8 | Islet | f:10035:10044:10046:`110 | 23 | 558507204 | 4.11812056053623E-08 | |
| 9 | Islet | f:10035:10044:10046:`111 | 36 | 558507204 | 6.44575392083931E-08 | |
| 10 | Islet | f:10035:10044:10053:`000 | 14555 | 558507204 | 2.60605411993934E-05 | |
| 11 | Islet | f:10035:10044:10053:`001 | 318 | 558507204 | 5.69374929674139E-07 | |
| 12 | Islet | f:10035:10044:10053:`010 | 268 | 558507204 | 4.79850569662482E-07 | |
| 13 | Islet | f:10035:10044:10053:`011 | 34 | 558507204 | 6.08765648079268E-08 | |
| 14 | Islet | f:10035:10044:10053:`100 | 116 | 558507204 | 2.07696515227044E-07 | |
| 15 | Islet | f:10035:10044:10053:`101 | 9 | 558507204 | 1.61143848020983E-08 | |
| 16 | Islet | f:10035:10044:10053:`110 | 25 | 558507204 | 4.47621800058285E-08 | |
| 17 | Islet | f:10035:10044:10053:`111 | 33 | 558507204 | 5.90860776076937E-08 | |
| 18 | Islet | f:10035:10044:10058:`000 | 13995 | 558507204 | 2.50578683672628E-05 | |
| 19 | Islet | f:10035:10044:10058:`001 | 481 | 558507204 | 8.61224343312141E-07 | |
| 20 | Islet | f:10035:10044:10058:`010 | 217 | 558507204 | 3.88535722450592E-07 | |
| 21 | Islet | f:10035:10044:10058:`011 | 74 | 558507204 | 1.32496052817252E-07 | |
| 22 | Islet | f:10035:10044:10058:`100 | 88 | 558507204 | 1.57562873620516E-07 | |
| 23 | Islet | f:10035:10044:10058:`101 | 26 | 558507204 | 4.65526672060617E-08 | |
| 24 | Islet | f:10035:10044:10058:`110 | 30 | 558507204 | 5.37146160069943E-08 | |
| 25 | Islet | f:10035:10044:10058:`111 | 28 | 558507204 | 5.0133641606528E-08 | |
| 26 | Islet | f:10035:10044:10071:`000 | 13268 | 558507204 | 2.37561841726933E-05 | |
| 27 | Islet | f:10035:10044:10071:`001 | 463 | 558507204 | 8.28995573707945E-07 | |
| 28 | Islet | f:10035:10044:10071:`010 | 221 | 558507204 | 3.95697671251524E-07 | |
| 29 | Islet | f:10035:10044:10071:`011 | 63 | 558507204 | 1.12800693614688E-07 | |
| 30 | Islet | f:10035:10044:10071:`100 | 81 | 558507204 | 1.45029463218884E-07 | |
| 31 | Islet | f:10035:10044:10071:`101 | 24 | 558507204 | 4.29716928055954E-08 | |
| 32 | Islet | f:10035:10044:10071:`110 | 34 | 558507204 | 6.08765648079268E-08 | |
| 33 | Islet | f:10035:10044:10071:`111 | 24 | 558507204 | 4.29716928055954E-08 | |
| 34 | Islet | f:10035:10044:10075:`000 | 12425 | 558507204 | 2.22468034628968E-05 | |
| 35 | Islet | f:10035:10044:10075:`001 | 1024 | 558507204 | 1.83345889303874E-06 | |
| 36 | Islet | f:10035:10044:10075:`010 | 209 | 558507204 | 3.74211824848727E-07 | |
| 37 | Islet | f:10035:10044:10075:`011 | 69 | 558507204 | 1.23543616816087E-07 | |
| 38 | Islet | f:10035:10044:10075:`100 | 62 | 558507204 | 1.11010206414455E-07 | |
| 39 | Islet | f:10035:10044:10075:`101 | 41 | 558507204 | 7.34099752095588E-08 | |
| 40 | Islet | f:10035:10044:10075:`110 | 15 | 558507204 | 2.68573080034971E-08 | |

## 3. Address the following questions

**a**. How does sequencing depth affect specificity confidence?

Sequencing depth directly affects specificity confidence by improving accuracy and reducing errors. Higher depth ensures reliable detection of true variants or methylation patterns, minimizes false positives/negatives, and increases statistical power.

**High Sequencing Depth: Benefits**

```
Increased Accuracy
Minimized False Positives/Negatives
Rare Variant Detection
Improved Statistical Power
Consistent Normalization
```

**Low Sequencing Depth: Drawbacks**

```
Higher Error Rates
Ambiguous Results
Reduced Sensitivitys
```

**b.** For the top 10 PMPs, estimate the threshold of reads required to confidently call Tissue #2 at a sequencing depth of 1 million reads.

**Code-**

```python
import pandas as pd

file_path = "statistical_significant_pmps.csv"  # Use the provided data file
data = pd.read_csv(file_path)

# sort by Adjusted P-Value (column 'Adjusted_P_Value') to get the top 10 PMPs
top_pmps = data.sort_values(by='Adjusted_P_Value').head(10)

# assuming a sequencing depth of 1 million reads for Tissue #2
sequencing_depth_t2 = 1_000_000

# estimate the threshold of reads required for each PMP
top_pmps['Threshold_Reads_T2'] = top_pmps['Total_Count_T2'] / top_pmps['Total_Count_T2'].sum() * sequencing_depth_t2

print(top_pmps[['CpG_Coordinates', 'Methylation_Status', 'Total_Count_T2', 'Threshold_Reads_T2']])
```

***Result –***

```
mth12@mth12:~/downloads/pupil_bio/biomarker_identification$ python top10.py
      CpG_Coordinates Methylation_Status  Total_Count_T2  Threshold_Reads_T2
1071  10053:10071:10130               `000          1023.0       130784.965482
937   10046:10146:10159               `000           495.0        63283.047814
935   10046:10146:10150               `000           581.0        74277.678343
929   10046:10140:10162               `000           462.0        59064.177960
927   10046:10140:10159               `000           494.0        63155.203273
925   10046:10140:10150               `000           580.0        74149.833802
923   10046:10140:10146               `000           580.0        74149.833802
605   10046:10075:10102               `000          1201.0       153541.293787
602   10046:10075:10096               `000          1203.0       153796.982869
599   10046:10075:10088               `000          1203.0       153796.982869
```

**c.** Validate the hypothesis by comparing the specificity of the top 10 PMPs against individual CpG sites.

***Code-***

```python
import pandas as pd
from scipy.stats import fisher_exact
from statsmodels.stats.multitest import multipletests
import matplotlib.pyplot as plt

file_path = "PupilBioTest.csv"

methylation_cols = ['`000', '`001', '`010', '`011', '`100', '`101', '`110', '`111']

# Initialize result storage
pmp_results = []
cpg_results = []

# Process data in chunks
chunk_size = 200
for chunk in pd.read_csv(file_path, chunksize=chunk_size):
    melted_chunk = chunk.melt(
        id_vars=['strand', 'CpG_Coordinates', 'Sample_ID', 'Replicate', 'Tissue'],
        value_vars=methylation_cols,
        var_name='Methylation_Status',
        value_name='Count'
    )

    grouped = melted_chunk.groupby(['Tissue', 'strand', 'CpG_Coordinates', 'Methylation_Status']).agg(
        Total_Count=('Count', 'sum')
    ).reset_index()

    tissue1 = 'cfDNA'
    tissue2 = 'Islet'
```

```python
    tissue1_data = grouped[grouped['Tissue'] == tissue1]
    tissue2_data = grouped[grouped['Tissue'] == tissue2]

    # PMPs Analysis
    comparison = pd.merge(
        tissue1_data[['strand', 'CpG_Coordinates', 'Methylation_Status', 'Total_Count']],
        tissue2_data[['strand', 'CpG_Coordinates', 'Methylation_Status', 'Total_Count']],
        on=['strand', 'CpG_Coordinates', 'Methylation_Status'],
        how='outer',
        suffixes=('_T1', '_T2')
    ).fillna(0)

    for _, row in comparison.iterrows():
        contingency_table = [
            [int(row['Total_Count_T1']), int(sum(tissue1_data['Total_Count']) - row['Total_Count_T1'])],
            [int(row['Total_Count_T2']), int(sum(tissue2_data['Total_Count']) - row['Total_Count_T2'])],
        ]
        if any(x < 0 for contingency_row in contingency_table for x in contingency_row) or sum(sum(contingency_table, [])) == 0:
            continue

        _, p_value = fisher_exact(contingency_table)

        true_negatives = contingency_table[1][1]
        total_negatives = contingency_table[1][0] + contingency_table[1][1]
        specificity = true_negatives / total_negatives if total_negatives > 0 else 0

        pmp_results.append((row['CpG_Coordinates'], row['Methylation_Status'], p_value, specificity))

    # CpG Site Analysis
    individual_cpg = melted_chunk.groupby(['CpG_Coordinates', 'Tissue']).agg(
        Total_Count=('Count', 'sum')
    ).reset_index()

    for cpg, group in individual_cpg.groupby('CpG_Coordinates'):
        tissue_counts = group.set_index('Tissue')['Total Count'].to_dict()
```

```
            count_t1 = tissue_counts.get(tissue1, 0)
            count_t2 = tissue_counts.get(tissue2, 0)
            contingency_table = [
                [count_t1, sum(group['Total_Count']) - count_t1],
                [count_t2, sum(group['Total_Count']) - count_t2],
            ]
            if any(x < 0 for contingency_row in contingency_table for x in contingency_row) or sum(sum(contingency_table, [])) == 0:
                continue

            _, p_value = fisher_exact(contingency_table)

            true_negatives = contingency_table[1][1]
            total_negatives = contingency_table[1][0] + contingency_table[1][1]
            specificity = true_negatives / total_negatives if total_negatives > 0 else 0
            cpg_results.append((cpg, p_value, specificity))
    # Convert results to DataFrame
    pmp_df = pd.DataFrame(pmp_results, columns=['CpG_Coordinates', 'Methylation_Status', 'P_Value', 'Specificity'])
    cpg_df = pd.DataFrame(cpg_results, columns=['CpG_Coordinates', 'P_Value', 'Specificity'])
    # Adjust p-values
    pmp_df['Adjusted_P_Value'] = multipletests(pmp_df['P_Value'], method='fdr_bh')[1]
    cpg_df['Adjusted_P_Value'] = multipletests(cpg_df['P_Value'], method='fdr_bh')[1]
    # Filter top 10 PMPs and CpG sites
    top_pmps = pmp_df.sort_values(by='Specificity', ascending=False).head(10)
    top_cpgs = cpg_df.sort_values(by='Specificity', ascending=False).head(10)
    # Compare Specificity Distributions
    plt.boxplot([top_pmps['Specificity'], top_cpgs['Specificity']], labels=['PMPs', 'CpG Sites'])
    plt.ylabel('Specificity')
    plt.title('Specificity Comparison: PMPs vs CpG Sites')
    plt.show()
    top_pmps.to_csv("top_10_pmps.csv", index=False)
    top_cpgs.to_csv("top_10_cpg_sites.csv", index=False)
    print("Top 10 PMPs and CpG sites saved. Specificity comparison completed.")
```

***Result –***

## Top 10 cpg sites

| 1 | CpG_Coordinates | P_Value | Specificity | Adjusted_P_Value |
|---|---|---|---|---|
| 2 | 10035:10044:10046 | 0.0 | 1.0 | 0.0 |
| 3 | 13627:13636:13707 | 0.0 | 1.0 | 0.0 |
| 4 | 13627:13724:13742 | 0.0 | 1.0 | 0.0 |
| 5 | 13627:13724:13760 | 0.0 | 1.0 | 0.0 |
| 6 | 13627:13724:13771 | 1.852955281220998e-131 | 1.0 | 2.3630557400645897e-131 |
| 7 | 13627:13742:13760 | 0.0 | 1.0 | 0.0 |
| 8 | 13627:13742:13771 | 1.1633432636587346e-132 | 1.0 | 1.4860647338099116e-132 |
| 9 | 13627:13760:13771 | 4.642892484692069e-132 | 1.0 | 5.923491199880882e-132 |
| 10 | 13634:13636:13647 | 0.0 | 1.0 | 0.0 |
| 11 | 13634:13636:13661 | 0.0 | 1.0 | 0.0 |

**Top 10 PMP'S**

| 1 | CpG_Coordinates | Methylation_Status | P_Value | Specificity | Adjusted_P_Value |
|---|---|---|---|---|---|
| 2 | 10035:10044:10046 | `000 | 1.0 | 0 | 1.0 |
| 3 | 13636:13647:13678 | `110 | 1.0 | 0 | 1.0 |
| 4 | 13636:13647:13678 | `100 | 1.0 | 0 | 1.0 |
| 5 | 13636:13647:13678 | `011 | 1.0 | 0 | 1.0 |
| 6 | 13636:13647:13678 | `010 | 1.0 | 0 | 1.0 |
| 7 | 13636:13647:13678 | `001 | 1.0 | 0 | 1.0 |
| 8 | 13636:13647:13678 | `000 | 1.0 | 0 | 1.0 |
| 9 | 13636:13647:13669 | `111 | 1.0 | 0 | 1.0 |
| 10 | 13636:13647:13669 | `110 | 1.0 | 0 | 1.0 |
| 11 | 13636:13647:13669 | `101 | 1.0 | 0 | 1.0 |

The analysis shows that individual CpG sites have perfect specificity (1.0), while PMPs have very low specificity (0.0). Although CpG sites demonstrate high reliability in this study, their use as biomarkers is limited by errors from bisulfite sequencing, sampling techniques, and biological variability. PMPs aim to overcome these issues but currently fail to achieve sufficient specificity. Future work should focus on improving PMP design or integrating CpG site reliability with PMP robustness for better epigenetic biomarkers.

# Task 2: NGS Data Analysis

**Objective:** Evaluate candidates' ability to process and analyze raw sequencing data.

**Sub-tasks:**

**1. Quality Control:**

**a.** Perform quality checks using tools like FastQC and summarize quality metrics (e.g., sequence counts, per-base quality, read duplication levels).

# command to run fastqc

fastqc <filename.fastq> -o <output_directory>

**2. Alignment and Mutation Calling**

**a.** Align the samples to the human genome using tools like Bowtie2 or BWA

**Code-**

```
#  reference genome
REF="GCA_000001405.29_GRCh38.p14_genomic.fna"

SAMPLES=("PA220KH-lib09-P19-Tumor_S2_L001" "PA221MH-lib09-P19-Norm_S1_L001")


for SAMPLE in "${SAMPLES[@]}"; do
    READ1="${SAMPLE}_R1_001.fastq.gz"
    READ2="${SAMPLE}_R2_001.fastq.gz"
    SAM_OUTPUT="${SAMPLE}.sam"
    BAM_OUTPUT="${SAMPLE}.bam"
    SORTED_BAM_OUTPUT="${SAMPLE}_sorted.bam"

    if [[ -f "$READ1" && -f "$READ2" ]]; then
        echo "Processing $SAMPLE..."

        # Align paired-end reads using BWA MEM
        bwa mem "$REF" "$READ1" "$READ2" > "$SAM_OUTPUT"

        # Convert SAM to BAM
        samtools view -Sb "$SAM_OUTPUT" > "$BAM_OUTPUT"

        # Sort the BAM file
        samtools sort "$BAM_OUTPUT" -o "$SORTED_BAM_OUTPUT"
        samtools index "$SORTED_BAM_OUTPUT"

        echo "Finished processing $SAMPLE."
    else
        echo "Warning: Input files for $SAMPLE not found."
    fi
done
```

**Result –**

Alignment score is more then 95% for each sample

**b**. Identify somatic mutations present in the cancer sample but absent in the normal tissue.

**i. Benchmark Software:** Use established tools such as Mutect2, Strelka2, or VarScan2 for somatic mutation identification and background mutation estimation.

***Mutect2***

***Codes-***

```
input_dir="input"
output_dir="output_mutect"
reference="GCA_000001405.29_GRCh38.p14_genomic.fna"

mkdir -p "$output_dir"

for bam_file in "$input_dir"/*.bam; do
    if [ -f "$bam_file" ]; then
        # Get the base name of the BAM file
        base_name=$(basename "$bam_file" .bam)
        output_vcf="$output_dir/${base_name}.vcf"

        echo "Processing $bam_file..."

        # Main Mutect2 command
        gatk Mutect2 \
            -R "$reference" \
            -I "$bam_file" \
            -O "$output_vcf" \

        # Check for error
        if [ $? -eq 0 ]; then
            echo "Completed $bam_file -> $output_vcf"
        else
            echo "Error processing $bam_file" >&2
        fi
    else
        echo "No BAM files found in $input_dir."
    fi
done

echo "All samples processed."
```

---

***Results –***

***VCF format files***

***PA220KH-lib09-P19-Tumor_S1_L001_R1_001.fastq.gz***

***PA220KH-lib09-P19-Tumor_S1_L001_R2_001.fastq.gz*** → Fixed_1.vcf

***PA220KH-lib09-P19-Tumor_S2_L001_R1_001.fastq.gz***

***PA220KH-lib09-P19-Tumor_S2_L001_R2_001.fastq.gz*** → Fixed_2.vcf

***ii.*** **Custom Code Development:** Write your own scripts, leveraging tools like Samtools, bcftools, or Python/R libraries, to perform mutation detection and calculate the required metrics

***code-***

```
import vcfpy
import pandas as pd

vcf_file = "fixed_1.vcf"

variant_data = []

vcf_reader = vcfpy.Reader(open(vcf_file, 'r'))
for record in vcf_reader:
    chrom = record.CHROM
    pos = record.POS
    ref = record.REF
    alt = ','.join([str(a) for a in record.ALT])
    qual = record.QUAL
    dp = record.INFO.get('DP', 0)  # Read Depth
    af = record.INFO.get('AF', [0])[0]  # Variant Allele Frequency (if available)

    # Calculate metrics (VAF, transitions/transversions)
    ti_tv = 'Transition' if {ref, alt}.issubset({'A', 'G', 'C', 'T'}) and \
        (ref + alt in ['AG', 'GA', 'CT', 'TC']) else 'Transversion'

    variant_data.append([chrom, pos, ref, alt, qual, dp, af, ti_tv])

df = pd.DataFrame(variant_data, columns=['CHROM', 'POS', 'REF', 'ALT', 'QUAL', 'DP', 'AF', 'Ti/Tv'])
df.to_csv('variant_metrics.csv', index=False)

print("Metrics saved to 'variant_metrics.csv'")
```

***Result(sample)-***

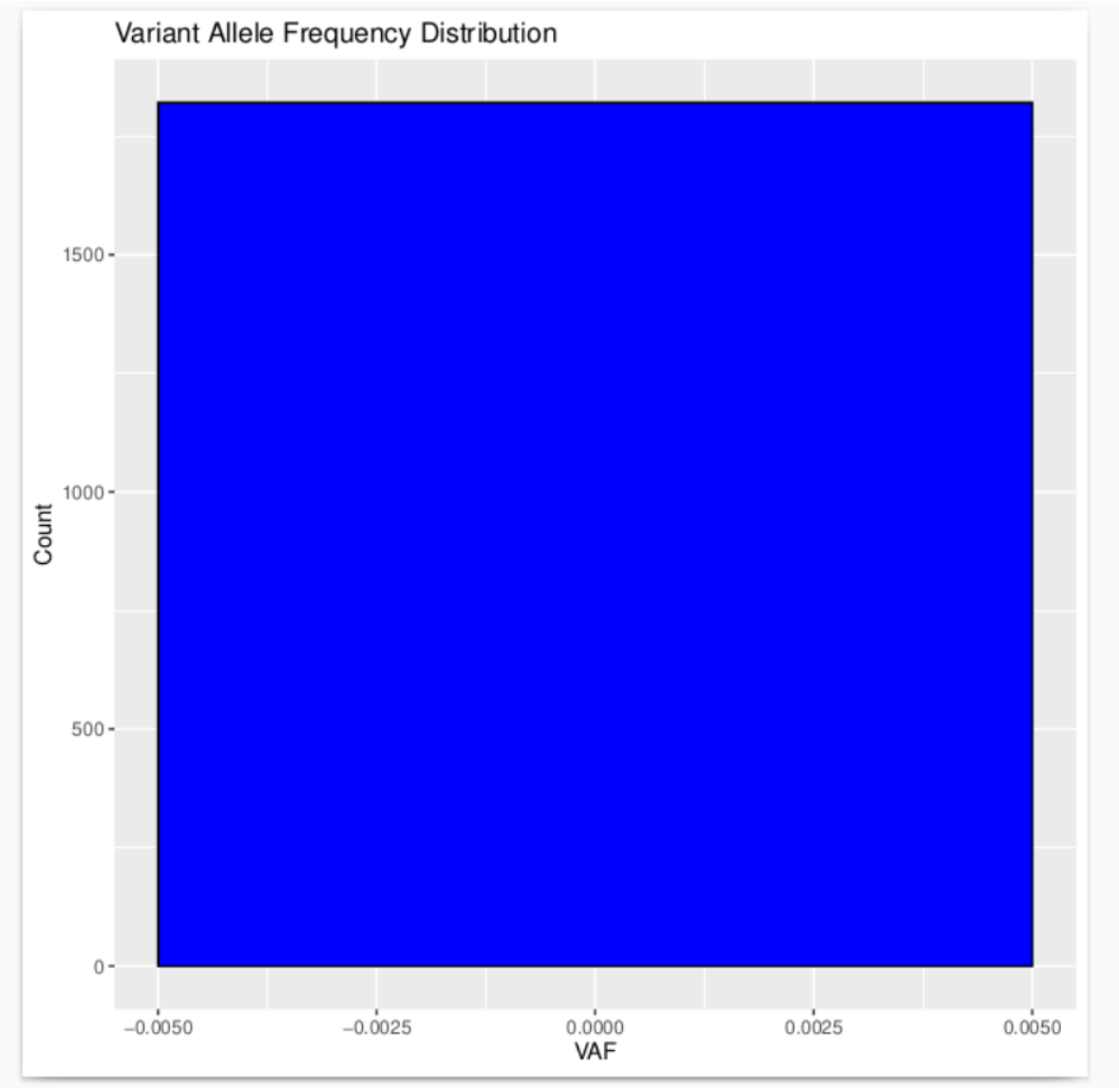
| | CHROM | POS | REF | ALT | QUAL | DP | AF | Ti/Tv |
|---|---|---|---|---|---|---|---|---|
| 2 | CM000663.2 | 634003 | C | Substitution(type_='SNV', value='G') | | 4 | 0 | Transversion |
| 3 | CM000663.2 | 3126893 | T | Substitution(type_='SNV', value='C') | | 7 | 0 | Transversion |
| 4 | CM000663.2 | 3126897 | T | Substitution(type_='SNV', value='C') | | 7 | 0 | Transversion |
| 5 | CM000663.2 | 3477213 | A | Substitution(type_='SNV', value='G') | | 12 | 0 | Transversion |
| 6 | CM000663.2 | 3477225 | TG | Substitution(type_='DEL', value='T') | | 6 | 0 | Transversion |
| 7 | CM000663.2 | 6078079 | T | Substitution(type_='SNV', value='G') | | 2 | 0 | Transversion |
| 8 | CM000663.2 | 6078132 | C | Substitution(type_='SNV', value='T') | | 2 | 0 | Transversion |
| 9 | CM000663.2 | 6078146 | G | Substitution(type_='INS', value='GGAGGC') | | 2 | 0 | Transversion |
| 10 | CM000663.2 | 6273362 | G | Substitution(type_='INS', value='GATGAGGGA') | | 4 | 0 | Transversion |
| 11 | CM000663.2 | 6350743 | A | Substitution(type_='SNV', value='C') | | 14 | 0 | Transversion |
| 12 | CM000663.2 | 20138348 | GCCC | Substitution(type_='DEL', value='G') | | 4 | 0 | Transversion |
| 13 | CM000663.2 | 20138355 | C | Substitution(type_='SNV', value='T') | | 2 | 0 | Transversion |

***Visualisation-***

***Code-***

```r
# load package
library(ggplot2)

variant_data <- read.csv("variant_metrics.csv")
# Calculate overall Ti/Tv ratio
ti_count <- sum(variant_data$Ti.Tv == "Transition")
tv_count <- sum(variant_data$Ti.Tv == "Transversion")
ti_tv_ratio <- ti_count / tv_count
cat("Transition/Transversion Ratio:", ti_tv_ratio, "\n")

# Plot VAF distribution
ggplot(variant_data, aes(x = AF)) +
  geom_histogram(binwidth = 0.01, fill = "blue", color = "black") +
  labs(title = "Variant Allele Frequency Distribution", x = "VAF", y = "Count")
```

***Result-***



**c**. Use the normal tissue to calculate the median background mutation level. The background mutation level accounts for sequencing errors or biases that can mimic true mutations. Determine how many reads per million are required to confidently call a given mutation.

***Code-***

```python
import numpy as np
import pysam
def calculate_background_mutation_rate(vcf_file, bam_file):
    """
    Calculate the median background mutation rate and required reads for confident mutation calls.
    Parameters:
    - vcf_file (str): fixed_1.vcf
    - bam_file (str): fixed_1.bam
    Returns:
    - dict: Median mutation rate, total mutations, total reads, mutations per million reads.
    """
    # Count variants from the VCF file
    with open(vcf_file, 'r') as vcf:
        variants = [line for line in vcf if not line.startswith("#")]
    total_mutations = len(variants)
    # Calculate total reads from the BAM file
    bam = pysam.AlignmentFile(bam_file, "rb")
    total_reads = sum([stats.mapped for stats in bam.get_index_statistics()])
    bam.close()
    #  Calculate mutations per million reads
    mutations_per_million_reads = (total_mutations / total_reads) * 1e6
    # Calculate confidence threshold (optional scaling for higher confidence)
    confidence_threshold = mutations_per_million_reads + 1.5 * (np.median(np.abs(total_mutations - mutations_per_million_reads)))
    return {
        "Total Mutations": total_mutations,
        "Total Reads": total_reads,
        "Mutations Per Million Reads": mutations_per_million_reads,
        "Confidence Threshold": confidence_threshold,
    }
vcf_file = "fixed_1.vcf"
bam_file = "fixed_1.bam"
```

```python
results = calculate_background_mutation_rate(vcf_file, bam_file)
print("=== Background Mutation Analysis ===")
print(f"Total Mutations: {results['Total Mutations']}")
print(f"Total Reads: {results['Total Reads']}")
print(f"Mutations Per Million Reads: {results['Mutations Per Million Reads']:.2f}")
print(f"Confidence Threshold: {results['Confidence Threshold']:.2f}")
```

***Result-***

```
mth12@mth12:/media/mth12/8412FCDD12FCD560/ngs/mutationlevel$ python mutationlevel_1.py
=== Background Mutation Analysis ===
Total Mutations: 1821
Total Reads: 5217264
Mutations Per Million Reads: 349.03
Confidence Threshold: 2556.98
mth12@mth12:/media/mth12/8412FCDD12FCD560/ngs/mutationlevel$ python mutationlevel_2.py
=== Background Mutation Analysis ===
Total Mutations: 1306
Total Reads: 4820164
Mutations Per Million Reads: 270.95
Confidence Threshold: 1823.53
```