Rx Only

cobas® EZH2 Mutation Test

For in vitro diagnostic use

IVD

cobas® EZH2 Mutation Test	24 Tests	M/N: 07258321190

Refer to the **cobas®** DNA Sample Preparation Kit (M/N 05985536190) for sample preparation.

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Intended use

The **cobas**® EZH2 Mutation Test is a real-time allele-specific PCR test for qualitative detection of single nucleotide mutations for Y646N, Y646F or Y646X (Y646H, Y646S, or Y646C), A682G, and A692V of the EZH2 gene in DNA extracted from formalin fixed paraffin embedded (FFPE) human follicular lymphoma tumor tissue specimens. The **cobas**® EZH2 Mutation Test is intended for the identification of follicular lymphoma patients with an EZH2 mutation for treatment with TAZVERIK™ (tazemetostat), in accordance with the approved therapeutic product labeling.

Specimens are processed using the **cobas**® DNA Sample Preparation Kit for manual sample preparation and the **cobas z** 480 analyzer for automated amplification and detection.

Summary and explanation of the test

Background

Non-Hodgkin Lymphoma is one of the most common cancers in the world, comprising nearly 3% of all cancers globally.[1] Follicular lymphoma (FL) is an indolent subtype of NHL and accounts for approximately 35% of all B cell lymphomas.[2] Several gene mutations in B-cells can change the gene expression profile and promote neoplastic transformation. This includes mutated forms of the enhancer of zeste 2 polycomb repressive complex 2 (EZH2) gene.[3] Somatic mutations in EZH2 can cause epigenetic and transcriptional modifications. The most prevalent mutations in FL are at codon Y646, followed by mutations at codons A682 and A692 of the EZH2 gene and are identified in up to 25% of patients with FL.[4,5]

The mutations detected by the **cobas**® EZH2 Mutation Test (**cobas** EZH2 Test) are listed in Table 1.

Table 1: Mutations detected by the cobas EZH2 Test

Exon	EZH2 Mutation	Nucleotide Nomenclature	Protein Nomenclature	COSMIC ID
16	Y646N	c.1936T>A	p.Tyr646Asn	COSM37031
16	Y646H	c.1936T>C	p.Tyr646His	COSM37030
16	Y646F	c.1937A>T	p.Tyr646Phe	COSM37028
16	Y646S	c.1937A>C	p.Tyr646Ser	COSM37029
16	Y646C	c.1937A>G	p.Tyr646Cys	COSM37032
18	A682G	c.2045C>G	p.Ala682Gly	COSM220386
18	A692V	c.2075C>T	p.Ala692Val	COSM220529

Principles of the procedure

The **cobas** EZH2 Test is based on two major processes: (1) manual specimen preparation to obtain DNA from Formalin Fixed Paraffin Embedded Tissue (FFPET); and (2) PCR amplification and detection of target DNA using complementary primer pairs and oligonucleotide probes labeled with fluorescent dyes. Mutation detection is achieved through PCR analysis with the **cobas z** 480 analyzer. A mutant control and negative control are included in each run to confirm the validity of the run.

Sample preparation

FFPET specimens are processed and genomic DNA isolated using the **cobas**® DNA Sample Preparation Kit for manual specimen preparation based on nucleic acid binding to glass fibers. A deparaffinized 5-µm section of an FFPET specimen is lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released genomic DNA from DNases. Subsequently, isopropanol is added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the genomic DNA is bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and then eluted with an aqueous solution. The amount of genomic DNA is determined by spectrophotometer and adjusted to a fixed concentration to be added to the amplification/detection mixture. The target DNA is then amplified and detected on the **cobas z** 480 analyzer using the amplification and detection reagents provided in the **cobas** EZH2 Test.

PCR amplification

Target selection

The **cobas** EZH2 Test uses primers that define specific base-pair sequences for each of the targeted mutations with base-pair sequences ranging from 94 to 104 base pairs long in exons 16 and 18 and 100 base pairs for the internal control in exon 11. Amplification occurs only in the regions of the EZH2 gene between the primers; the entire EZH2 gene is not amplified.

Target amplification

A derivative of *Thermus* species Z05-AS1 DNA polymerase is utilized for target amplification. First, the PCR mixture is heated to denature the genomic DNA and expose the primer target sequences. As the mixture cools, the upstream and downstream primers anneal to the target DNA sequences. The Z05-AS1 DNA polymerase, in the presence of divalent metal ion and excess dNTP, extends each annealed primer, thus synthesizing a second DNA strand. This completes the first cycle of PCR, yielding a double-stranded DNA copy which includes the targeted base-pair regions of the EZH2 gene. This process is repeated for a number of cycles, with each cycle effectively doubling the amount of amplicon DNA.

Automated real-time mutation detection

The **cobas** EZH2 Test utilizes real-time PCR technology. Each target-specific, oligonucleotide probe in the reaction is labeled with a fluorescent dye that serves as a reporter, and with a quencher molecule that absorbs (quenches) fluorescent emissions from the reporter dye within an intact probe. During each cycle of amplification, probe complementary to the

single-stranded DNA sequence in the amplicon binds and is subsequently cleaved by the 5' to 3' nuclease activity of the Z05-AS1 DNA Polymerase. Once the reporter dye is separated from the quencher by this nuclease activity, fluorescence of a characteristic wavelength can be measured when the reporter dye is excited by the appropriate spectrum of light. Four different reporter dyes are used to detect the EZH2 sequences targeted by the test. Amplification of the targeted EZH2 sequences are detected independently across three reactions by measuring fluorescence at the four characteristic wavelengths in dedicated optical channels.

Selective amplification

Selective amplification of target nucleic acid from the sample is achieved in the **cobas** EZH2 Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP).[6] The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing thymidine. Deoxyuridine is not present in naturally occurring DNA but is always present in amplicon due to the use of dUTP in place of deoxythymidine triphosphate as one of the nucleotide triphosphates in the Master Mix reagents; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by AmpErase enzyme prior to amplification of the target DNA. The AmpErase enzyme, which is included in the Master Mix reagents, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step at alkaline pH, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme is inactive at temperatures above 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon.

MATERIALS AND REAGENTS

Materials and reagents provided

Kit/Cassettes	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning[a]
cobas® EZH2 Mutation Test 24 Tests (M/N: 07258321190)	**EZH2 MMX-1** (EZH2 Master Mix 1) (M/N 7258283001) Tricine Potassium chloride Potassium hydroxide Glycerol Tween 20 EDTA 0.09% Sodium azide Dimethyl sulfoxide < 0.10% dNTPs < 0.15% Enzyme Z05-AS1 DNA polymerase (microbial) < 0.01% aptamer < 0.01% AmpErase (uracil-N-glycosylase) enzyme (microbial) < 0.01% Upstream and downstream EZH2 primers < 0.01% Fluorescent labeled EZH2 probes	2 x 0.48 mL	N/A
	EZH2 MMX-2 (EZH2 Master Mix 2) (M/N 7258291001) Tricine Potassium chloride Potassium hydroxide Glycerol Tween 20 EDTA 0.09% Sodium azide Dimethyl sulfoxide < 0.10% dNTPs < 0.15% Enzyme Z05-AS1 DNA polymerase (microbial) < 0.01% aptamer < 0.01% AmpErase (uracil-N-glycosylase) enzyme (microbial) < 0.01% Upstream and downstream EZH2 primers < 0.01% Fluorescent labeled EZH2 probes	2 x 0.48 mL	N/A
	EZH2 MMX-3 (EZH2 Master Mix 3) (M/N 7258305001) Tricine Potassium chloride Potassium hydroxide Glycerol Tween 20 EDTA 0.09% Sodium azide Dimethyl sulfoxide < 0.10% dNTPs < 0.15% Enzyme Z05-AS1 DNA polymerase (microbial) < 0.01% aptamer < 0.01% AmpErase (uracil-N-glycosylase) enzyme (microbial) < 0.01% Oligonucleotides < 0.01% Upstream and downstream EZH2 primers < 0.01% Fluorescent labeled EZH2 probes	2 x 0.48 mL	N/A

Kit/Cassettes	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning[a]
cobas® **EZH2 Mutation Test** 24 Tests (M/N: 07258321190)	**MGAC** (Magnesium acetate) (M/N 05854326001) Magnesium acetate 0.09% Sodium azide	6 x 0.20 mL	N/A
	EZH2 MC (EZH2 Mutant Control) (M/N 7258313001) Tris base Trizma HCl EDTA Poly-rA RNA (synthetic) <0.05% Sodium azide < 0.01% EZH2 wild-type DNA (cell culture) < 0.01% EZH2 Plasmid DNA	6 x 0.10 mL	N/A
	DNA SD (DNA Specimen Diluent) (M/N 05854474001) Tris-HCl buffer 0.09% Sodium azide	2 x 3.5 mL	N/A

[a] Product safety labeling primarily follows EU GHS guidance

Reagent storage and handling

Reagent	Storage Temperature	Storage Time
cobas® EZH2 Mutation Test*	2°C to 8°C	Once opened, stable for 4 uses up to 90 days or until the expiration date indicated, whichever comes first.

*** EZH2 MMX-1, EZH2 MMX-2, EZH2 MMX-3**, and working MMX (prepared by the addition of **MGAC** to **EZH2 MMX-1 or EZH2 MMX-2** or **EZH2 MMX-3**) should be protected from prolonged exposure to light. Working MMX must be stored at 2ºC to 8ºC in the dark. The prepared samples and controls must be added within 1 hour of preparation of the working MMX. Amplification must be started within 1 hour from the time that the processed samples and controls are added to the working MMX.

Additional materials required but not provided

Materials
cobas® DNA Sample Preparation Kit (Roche M/N 05985536190)
Bleach
70% Ethanol
cobas® 4800 System Microwell Plate (AD-plate) and sealing film (M/N Roche 05232724001)
cobas® 4800 System sealing film applicator (supplied with the installation of the **cobas**® 4800 System) (Roche M/N 04900383001)
Adjustable pipettors* (Capable of pipetting 5-1000 µL)
Aerosol barrier or positive displacement DNase-free tips
Bench top microcentrifuge* (capable of 20,000 x g)
Locking-lid microcentrifuge tubes (1.5 mL sterile, RNase/DNase free, PCR grade)
Microcentrifuge tube racks
Freezer capable of -25ºC to -15ºC storage
Refrigerator capable of 2ºC to 8ºC storage
Spectrophotometer for measuring DNA concentration*
Vortex mixer*
Disposable gloves, powder-free

* All equipment should be maintained according to the manufacturer's instructions.

For more information regarding the materials sold separately, contact your local Roche representative.

Instrumentation and software required

Required Instrumentation and Software, Not Provided	M/N
cobas z 480 Analyzer and Control Unit	05200881001
cobas® 4800 System Application Software (Core) version 2.1 or higher	07170114001
cobas® EZH2 Analysis Package Software version 1.0 or higher	07371969001

For more information regarding the materials sold separately, contact your local Roche representative.

Precautions and handling requirements

Warnings and precautions

As with any test procedure, good laboratory practice is essential to the proper performance of this assay.

- For *in vitro* diagnostic use only.
- Safety Data Sheets (SDS) are available upon request from your local Roche office.
- This test is for use with FFPET samples. Samples should be handled as if infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories[7] and in the CLSI Document M29-A4.[8]
- The use of sterile disposable pipettes and DNase-free pipettor tips is recommended.

Good laboratory practice

- Do not pipette by mouth.
- Do not eat, drink or smoke in laboratory work areas.
- Wash hands thoroughly after handling samples and kit reagents.
- Wear eye protection, laboratory coats and disposable gloves when handling any reagents. Avoid contact of these materials with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills occur, dilute with water before wiping dry.
- Thoroughly clean and disinfect all laboratory work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in distilled or deionized water (dilute household bleach 1:10). Follow by wiping the surface with 70% ethanol.

Note: *Commercial liquid household bleach typically contains sodium hypochlorite at a concentration of 5.25%. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.*

Contamination

- Gloves must be worn and must be changed between handling samples and **cobas** EZH2 Test reagents to prevent contamination. Avoid contaminating gloves when handling samples.
- Gloves must be changed frequently to reduce the potential for contamination.
- Gloves must be changed before leaving DNA Isolation areas or if contact with solutions or a sample is suspected.
- Avoid microbial and ribonuclease contamination of reagents.
- The amplification and detection work area should be thoroughly cleaned before working MMX preparation. Supplies and equipment should be dedicated to each activity and not used for other activities or moved between areas. For example, pipettors and supplies used for DNA Isolation must not be used to prepare reagents for amplification and detection.
- It is highly recommended that workflow in the laboratory proceed in a uni-directional manner, completing one activity before proceeding to the next activity. For example, DNA isolation should be completed before starting amplification and detection. DNA isolation should be performed in an area separate from amplification and detection. To avoid contamination of the working master mix with DNA samples, the amplification and detection work area should be thoroughly cleaned before working master mix preparation.

Integrity

- Do not use kits after their expiration dates.
- Do not pool reagents from different kits or lots.
- Do not use disposable items beyond their expiration date.
- All disposable items are for one time use. Do not reuse.
- All equipment should be properly maintained according to the manufacturer's instructions.

Disposal

- **MGAC**, **EZH2 MMX-1**, **EZH2 MMX-2**, **EZH2 MMX-3**, **EZH2 MC**, and **DNA SD** contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of cold water to prevent azide buildup.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

Spillage and cleaning

- If spills occur on the **cobas**® 4800 instrument, follow the instructions in the **cobas**® 4800 System - User Assistance to clean.
- Do not use sodium hypochlorite solution (bleach) for cleaning the **cobas z** 480 analyzer. Clean the **cobas z** 480 analyzer according to procedures described in the **cobas**® 4800 System - User Assistance.
- For additional warnings, precautions and procedures to reduce the risk of contamination for the **cobas z** 480 analyzer, consult the **cobas**® 4800 System - User Assistance.

Sample collection, transport, and storage

Note: *Handle all specimens as if they are capable of transmitting infectious agents.*

Sample collection

FFPET specimens have been verified for use with the **cobas** EZH2 Test.

Sample transport, storage, and stability

FFPET specimens can be transported at 15°C to 30°C. Transportation of FFPET specimens must comply with country, federal, state, and local regulations for the transport of etiologic agents.[9]

FFPET specimens may be stored at 15°C to 30°C for up to 12 months after the date of collection. Five-µm sections may be stored at 15°C to 30°C for up to 60 days.

FFPET specimens are stable for either:

FFPET Specimen Type	FFPET Block	5-µm FFPET Section
FFPET Sample Storage Temperature	15°C to 30°C	15°C to 30°C
Storage Time	Up to 12 months	Up to 60 days

Processed sample storage and stability

Processed samples (extracted DNA) are stable for one of the following:

Extracted DNA Storage Temperature	-15°C to -25°C	2°C to 8°C	15°C to 30°C
Storage Time	Up to 3 freeze thaws over 60 days	Up to 14 days	Up to 1 day

Extracted DNA should be used within the recommended storage periods or before the expiration date of the **cobas**® DNA Sample Preparation Kit used to extract the DNA, whichever comes first.

Test procedure

Running the test

Figure 1: cobas EZH2 Test workflow with cobas® DNA Sample Preparation Kit

1	Start the system
2	Perform instrument maintenance
3	Remove samples and reagents from storage
4	Deparaffinize samples
5	Perform DNA isolation
6	Elute DNA
7	Create work order and print plate layout
8	Prepare amplification reagents
9	Load AD-plate with amplification reagents
10	Load AD-plate with sample
11	Seal AD-plate
12	Load AD-plate on the **cobas z** 480 analyzer
13	Start the run
14	Review results
15	With LIS: send results to LIS
16	Unload analyzer

Instructions for use

Note: *Only FFPET sections of 5-µm thickness containing at least 15% tumor content by area are to be used in the **cobas** EZH2 Test. Any sample containing less than 15% tumor content by area should be macro-dissected after deparaffinization.*

Note: *Refer to the **cobas**® 4800 System – User Assistance for detailed operating instructions for the **cobas z** 480 analyzer.*

Run size

A single run can include from 1 to 30 samples (plus controls) per 96-well microwell plate. When running more than 24 samples, multiple **cobas** EZH2 Test kits will be required.

The **cobas** EZH2 Test contains sufficient reagents for 8 runs of 3 samples (plus controls) for a maximum of 24 samples per kit.

Full process controls

This test requires a full-process negative control. For each run, process a negative control concurrently with the sample(s) beginning with the DNA isolation procedure.

DNA isolation

DNA is isolated from FFPET specimens using the **cobas**® DNA Sample Preparation Kit.

Macro-dissection

If the sample contains less than 15% tumor content by area, the sample must be macro-dissected as part of the sample preparation.

DNA quantitation

Note: *Measurement of DNA concentration should be performed immediately after the DNA Isolation procedure and prior to storage.*

Note: *Store DNA Stock according to instructions in* ***Sample transport, storage, and stability*** *section.*

1. Mix each DNA Stock by vortexing for 5 seconds.
2. Quantify DNA using a spectrophotometer according to the manufacturer's protocol. Use **DNA EB** as the blank for the instrument. An average of two consistent readings is necessary. The two measurements should be within ± 10% of each other when the DNA concentration readings are ≥ 20.0 ng/µL. For DNA concentration readings < 20.0 ng/µL, the two measurements should be within ± 2 ng/µL. If the two measurements are not within ± 10% of each other when the DNA concentration readings are ≥ 20.0 ng/µL or within ± 2 ng/µL when the DNA concentration readings are < 20.0 ng/µL, an additional 2 readings must be taken until the requirements are met. The average of these two new measurements should then be calculated.

Note: *The DNA Stock from the processed negative control* ***(NEG)*** *does not need to be measured.*

3. The DNA Stock concentration from the samples must be ≥ 2 ng/µL to perform the **cobas** EZH2 Test. Three amplification/detections are run per sample, using 25 µL of a 2 ng/µL dilution of DNA Stock (total of 50 ng DNA) for each amplification/detection.

Note: *Each DNA Stock must have a minimum concentration of 2 ng/µL to perform the* ***cobas*** *EZH2 Test. If the concentration of a DNA Stock is < 2 ng/µL, repeat the deparaffinization, DNA Isolation, and DNA Quantitation procedures for that sample using two 5-µm FFPET sections. For mounted samples, after deparaffinization, combine the tissue from both sections into one tube. Immerse the tissue in DNA TLB + PK and perform DNA isolation and quantitation as described above. For unmounted samples, combine the tissue from both sections into one tube and perform deparaffinization. Immerse the tissue in DNA TLB + PK and perform DNA isolation and quantitation as described above. If the DNA Stock is still < 2 ng/µL, request another FFPET sample section from the referring clinical site.*

Amplification and detection

Note: *To avoid contamination of working MMX with DNA samples, amplification and detection should be performed in an area separated from DNA Isolation. The amplification and detection work area should be thoroughly cleaned before working MMX preparation. For proper cleaning, all surfaces including racks and pipettors should be thoroughly wiped with 0.5% sodium hypochlorite solution followed by wiping with a 70% ethanol solution. Commercial liquid household bleach typically contains sodium hypochlorite at a concentration of 5.25%. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.*

Instrument set-up

Refer to the **cobas**® 4800 System - User Assistance for detailed instruction for the **cobas z** 480 set up.

Test order set-up

For detailed instructions on the **cobas** EZH2 Test workflow steps, refer to the **cobas**® 4800 System - User Assistance.

Generate a plate map with the position of all the samples and controls in the run. The Mutant Control is loaded into positions A01 – A03 on the plate. The Negative Control is loaded into positions B01 – B03 on the plate. Diluted samples are then added in sets of 3 columns, starting from C01 – C03 through H10 – H12, as shown in Figure 2.

Figure 2: Plate layout for the cobas EZH2 Test

Row / Column	01	02	03	04	05	06	07	08	09	10	11	12
A	MC MMX 1	MC MMX 2	MC MMX 3	S7 MMX 1	S7 MMX 2	S7 MMX 3	S15 MMX 1	S15 MMX 2	S15 MMX 3	S23 MMX 1	S23 MMX 2	S23 MMX 3
B	NEG MMX 1	NEG MMX 2	NEG MMX 3	S8 MMX 1	S8 MMX 2	S8 MMX 3	S16 MMX 1	S16 MMX 2	S16 MMX 3	S24 MMX 1	S24 MMX 2	S24 MMX 3
C	S1 MMX 1	S1 MMX 2	S1 MMX 3	S9 MMX 1	S9 MMX 2	S9 MMX 3	S17 MMX 1	S17 MMX 2	S17 MMX 3	S25 MMX 1	S25 MMX 2	S25 MMX 3
D	S2 MMX 1	S2 MMX 2	S2 MMX 3	S10 MMX 1	S10 MMX 2	S10 MMX 3	S18 MMX 1	S18 MMX 2	S18 MMX 3	S26 MMX 1	S26 MMX 2	S26 MMX 3
E	S3 MMX 1	S3 MMX 2	S3 MMX 3	S11 MMX 1	S11 MMX 2	S11 MMX 3	S19 MMX 1	S19 MMX 2	S19 MMX 3	S27 MMX 1	S27 MMX 2	S27 MMX 3
F	S4 MMX 1	S4 MMX 2	S4 MMX 3	S12 MMX 1	S12 MMX 2	S12 MMX 3	S20 MMX 1	S20 MMX 2	S20 MMX 3	S28 MMX 1	S28 MMX 2	S28 MMX 3
G	S5 MMX 1	S5 MMX 2	S5 MMX 3	S13 MMX 1	S13 MMX 2	S13 MMX 3	S21 MMX 1	S21 MMX 2	S21 MMX 3	S29 MMX 1	S29 MMX 2	S29 MMX 3
H	S6 MMX 1	S6 MMX 2	S6 MMX 3	S14 MMX 1	S14 MMX 2	S14 MMX 3	S22 MMX 1	S22 MMX 2	S22 MMX 3	S30 MMX 1	S30 MMX 2	S30 MMX 3

Where: MC= Mutant Control, NEG = Negative Control S# = sample ID, and MMX # corresponds to Master Mix Reagent 1, 2, or 3.

Note: *Any given sample must be spread across three consecutive columns in one row in order to generate a response.*

Note: *Working Master Mix 1 must be loaded into column 01, 04, 07, and 10 on the plate. Working Master Mix 2 must be loaded into column 02, 05, 08, and 11 on the plate. Working Master Mix 3 must be loaded into column 03, 06, 09, and 12 on the plate.*

Note: *Up to 30 samples can be loaded onto a single plate. If more than one reagent kit is required to process all of the samples on the plate, then the kits must all be from the same lot.*

Dilution calculation of sample DNA stock

Dilution calculation for DNA stock concentrations from 2 ng/µL to 36 ng/µL

Note: *DNA stocks from samples should be diluted immediately prior to amplification and detection.*

Note: *Three amplification/detections are run for each sample requiring a total volume of 75 µL (25 µL for each of three reactions) of a 2 ng/µL dilution of DNA Stock (total of 150 ng DNA).*

1. For each sample, calculate the volume (µL) of DNA stock needed:

   µL of DNA stock = (90 µL x 2 ng/µL) ÷ DNA Stock concentration [ng/µL]

2. For each sample, calculate the volume (µL) of DNA Specimen Diluent (**DNA SD**) needed:

   µL of **DNA SD** = 90 µL – µL of DNA Stock

Example:

DNA stock concentration = 6.5 ng/µL

1. µL of DNA Stock = (90 µL x 2 ng/µL) ÷ 6.5 ng/µL = 27.7 µL
2. µL of **DNA SD** = (90 µL – 27.7 µL) = 62.3 µL

Dilution calculations for DNA stock concentrations > 36 ng/µL

Note: *DNA Stocks from samples should be diluted immediately prior to amplification and detection.*

Note: *Three amplification/detections are run for each sample requiring a total volume of 75 µL (25 µL for each of three reactions) of a 2 ng/µL dilution of DNA stock (total of 150 ng DNA).*

1. At DNA Stock concentrations > 36 ng/µL, use the following formula to calculate the amount of **DNA SD** required to prepare at least 90 µL of diluted DNA stock. This is to ensure that each sample uses a minimum of 5 µL of DNA stock.
2. For each sample, calculate the volume (µL) of **DNA SD** needed to dilute 5 µL of DNA stock to 2 ng/µL:

   Vol. of **DNA SD** required in µL = [(5 µL of DNA stock x DNA stock concentration in ng/µL) / 2 ng/µL] – 5 µL

Example:

DNA stock concentration = 100 ng/µL

1. Vol. of **DNA SD** required in µL = [(5 µL x 100 ng/µL) / 2 ng/µL] – 5 µL = 245 µL
2. Use the calculated volume of **DNA SD** to dilute 5 µL of DNA stock.

Sample dilution

1. Prepare the appropriate number of 1.5 mL locking-lid microcentrifuge tubes for DNA dilutions by labeling them with the proper sample identification.
2. Using a pipettor with an aerosol-resistant tip, pipette the calculated volumes of **DNA SD** into the respectively labeled tubes. Pipette 45 µL of **DNA SD** into a locking-lid microcentrifuge tube labeled as **NEG**.
3. Vortex each DNA stock and the negative control for 5 to 10 seconds.
4. Using a pipettor with an aerosol-resistant pipette tip (new tip for each pipetting), gently pipette the calculated volume of each DNA stock into the respective tube containing **DNA SD**. Pipette 45 µL of negative control (extracted eluate) into the **NEG** tube.
5. Cap the tubes and vortex each for 5 to 10 seconds.
6. Change gloves.

Reaction set-up

Preparation of working master mixes (MMX-1, MMX-2 and MMX-3)

Note: ***EZH2 MMX-1, EZH2 MMX-2, EZH2 MMX-3****, and working MMX are light-sensitive and must be protected from prolonged exposure to light.*

Note: *Due to the viscosity of the* ***EZH2 MMX*** *reagents and working MMX, pipette slowly to ensure all mix is completely dispensed from the tip.*

Note: *The* ***EZH2 MMX-1, EZH2 MMX-2,*** *and* ***EZH2 MMX-3*** *may appear light blue/purplish. This does not affect the performance of the reagent.*

Prepare three bulk working MMX, one containing **EZH2 MMX-1**, one containing **EZH2 MMX-2**, and the other containing **EZH2 MMX-3** in separate 1.5 mL locking-lid microcentrifuge tubes.

1. Calculate the volume of **EZH2 MMX-1** or **EZH2 MMX-2** or **EZH2 MMX-3** required for each working MMX using the following formula:

   Volume of **EZH2 MMX-1** or **EZH2 MMX-2** or **EZH2 MMX-3** required = (Number of Samples + 2 Controls +1) x 20 µL
2. Calculate the volume of **MGAC** required for each working MMX using the following formula:

   Volume of **MGAC** required = (Number of Samples + 2 Controls +1) x 5 µL

Use Table 2 to determine the volume of each reagent needed for the preparation of working MMX based on the number of samples included in the run.

Table 2: Volumes of reagents needed for working MMX-1, working MMX-2 and working MMX-3

		# of Samples*									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
MMX	20 µL	80	100	120	140	160	180	200	220	240	260
MGAC	5 µL	20	25	30	35	40	45	50	55	60	65
Total Vol. for Each Working MMX (µL)		100	125	150	175	200	225	250	275	300	325

* Volumes for # of Samples is based on the sum of the # Samples + 2 Controls + 1

3. Remove the appropriate number of **EZH2 MMX-1, EZH2 MMX-2, EZH2 MMX-3,** and **MGAC** vials from 2°C to 8°C storage. Vortex each reagent for 5 seconds and collect liquid at the bottom of the tube before use. Label a sterile microcentrifuge tube for working MMX-1, working MMX-2, and working MMX-3.
4. Add the calculated volume of **EZH2 MMX-1** or **EZH2 MMX-2** or **EZH2 MMX-3** to their respective working MMX tube.
5. Add the calculated volume of **MGAC** to the working MMX tubes.
6. Vortex the tubes for 3 to 5 seconds to ensure adequate mixing.

Note: *Samples and controls should be added to the AD-plate within 1 hour after the preparation of the working MMXs.*

Note: *Use only* ***cobas***® *4800 System AD-plate and Sealing film.*

Preparation of plate

1. Pipette 25 µL of working MMX into each reaction well of the AD-plate that is needed for the run. Do not allow the pipettor tip to touch the plate outside the well.
    - Add working MMX-1 (containing **EZH2 MMX-1**) to the AD-plate wells in columns 01, 04, 07, and 10, as needed.
    - Add working MMX-2 (containing **EZH2 MMX-2**) to the AD-plate wells in columns 02, 05, 08, and 11, as needed.
    - Add working MMX-3 (containing **EZH2 MMX-3**) to the AD-plate wells in columns 03, 06, 09, and 12, as needed.
2. Pipette 25 µL of **EZH2 MC** into wells A01, A02, and A03 of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.
3. Using a new pipettor tip, pipette 25 µL of **NEG** into wells B01, B02, and B03 of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.

Note: *Each run must contain **EZH2 MC** in wells A01, A02 and A03, and **NEG** in wells B01, B02, and B03 or the run will be invalidated by the **cobas z** 480 analyzer.*

Note: *Change gloves as needed to protect against sample-to-sample contamination and external PCR reaction tube contamination.*

4. Using new pipettor tips for each diluted sample DNA, add 25 µL of the first sample DNA to wells C01, C02, and C03 of the AD-plate, using a new tip for the addition of the sample DNA to each well; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for the diluted DNA from each sample and follow the template in Figure 2 until all samples' dilutions are loaded onto the AD-plate. Ensure that all liquid is collected at the bottom of the wells.
5. Cover the AD-plate with sealing film (supplied with the plates). Use the sealing film applicator to seal the film firmly to the AD-plate.
6. Confirm that all liquid is collected at the bottom of each well before starting PCR.

Note: *Amplification and detection should be started within 1 hour after the addition of the first sample DNA dilution to the working MMX.*

Starting PCR

Refer to the **cobas**® 4800 System - User Assistance for detailed instructions on the EZH2 workflow steps.

Results

Interpretation of results

Note: *All run and sample validation is performed by the* ***cobas***® *4800 software.*

Note: *A valid test run may include both valid and invalid sample results.*

For a valid run, sample results are interpreted as shown in Table 3.

Table 3: Result interpretation for the cobas EZH2 Test

Test Result	Mutation Result	Interpretation
Mutation Detected	Y646N A692V Y646F A682G Y646X (Y646H, Y646S, or Y646C) More than one mutation may be present	Mutation detected in specified targeted EZH2 region.
No Mutation Detected (NMD)*	N/A	Mutation not detected in targeted EZH2 regions.
Invalid	N/A	Sample result is invalid. Repeat the testing of samples with invalid results following the instructions outlined in the **Retesting of samples with invalid results** section below.
Failed	N/A	Failed run due to hardware or software failure. Contact your local Roche office for technical assistance.

* A "No Mutation Detected" result does not preclude the presence of a mutation in the targeted EZH2 regions because results depend on percent mutant sequences, adequate sample integrity, absence of inhibitors, and sufficient DNA to be detected.

Result flags may be found under the Result tab (screen) or Flags column (report). Refer to Result Flag section for more detail.

Retesting of samples with invalid results

1. Repeat dilution of the invalid sample DNA stock starting from "Dilution Calculation of Sample DNA Stock" and "Sample Dilution" procedures in the **Amplification and detection** section.
2. After performing the DNA stock dilution to 2 ng/µL as described in "Sample Dilution", continue with "Preparation of working master mixes" and the remainder of the amplification and detection procedure.

Note: *If the sample remains invalid after retesting or there was not enough DNA stock to prepare another dilution in Retesting of samples with invalid results, step 1, repeat the entire test procedure for that sample, starting with Deparaffinization and DNA isolation using a new 5-µm FFPET tumor section.*

Quality control and validity of results

One set of **cobas** EZH2 Test Mutant Control (**EZH2 MC**) (wells A01, A02 and A03) and negative control (**NEG**) (wells B01, B02 and B03) for working MMX-1, working MMX-2, and working MMX-3 are included in each run of up to 30 samples. A run is valid if the **EZH2 MC** and the **NEG** are valid. If an **EZH2 MC** or **NEG** is invalid, the entire run is invalid and must be repeated. Prepare a fresh dilution of the previously isolated sample DNA Stock to set up a new AD-plate with controls for amplification and detection.

Mutant control

The **EZH2 MC** result must be 'Valid'. If the **EZH2 MC** results are consistently invalid, contact your local Roche office for technical assistance.

Negative control

The **NEG** result must be 'Valid'. If the **NEG** results are consistently invalid, contact your local Roche office for technical assistance.

Procedural limitations

1. Test only the indicated specimen types. The **cobas** EZH2 Test was verified with NHL FFPET tumor specimens.
2. The **cobas** EZH2 Test has only been validated using the **cobas**® DNA Sample Preparation Kit.
3. Detection of a mutation is dependent on the number of copies present in the specimen and may be affected by sample integrity, amount of isolated DNA, and the presence of interfering substances.
4. Reliable results are dependent on adequate specimen fixation, transport, storage and processing. Follow the procedures **cobas**® DNA Sample Preparation Kit Instructions for Use, in this Instructions for Use, and in the **cobas**® 4800 System - User Assistance.
5. The effects of other potential variables such as specimen fixation variables have not been evaluated.
6. The addition of AmpErase enzyme into the **cobas** EZH2 Test Master Mix enables selective amplification of target DNA; however, good laboratory practices and careful adherence to the procedures specified in this Instructions for Use are necessary to avoid contamination of reagents.
7. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas**® 4800 System.
8. Only the **cobas z** 480 analyzer has been verified for use with this product. No other thermal cycler with real-time optical detection can be used with this product.
9. The presence of PCR inhibitors may cause false negative or invalid results.
10. Though rare, mutations within the genomic DNA regions of the EZH2 gene covered by the primers or probes used in the **cobas** EZH2 Test may result in failure to detect presence of a mutation.
11. The **cobas** EZH2 Test is validated for use with 50 ng of DNA per reaction well. DNA input amounts lower than 50 ng per reaction well are not recommended.
12. The **cobas** EZH2 Test is a qualitative test. The test is not for quantitative measurements of percent mutation.
13. FFPET specimens containing degraded DNA may affect the ability of the test to detect the EZH2 mutations.
14. Samples with results reported as "No Mutation Detected" may harbor EZH2 mutations not detected by the assay.
15. The **cobas** EZH2 Test detects EZH2 mutations in patients whose tumors have the exon 16 (Y646N, Y646F, Y646H, Y646S, and Y646C) substitutions and exon 18 (A682G and A692V) substitutions but not any other EZH2 mutations.

Non-clinical performance evaluation

Note: *The study descriptions below include cumulative data performed with the* ***cobas*** *EZH2 Test.*

For the non-clinical studies described below, percentage of tumor was assessed by pathology review. Next-generation sequencing (NGS) was used to select the specimens for testing. Follicular lymphoma is a type of Non-Hodgkin lymphoma. Due to the rarity of FL specimens, other types of NHL were used to supplement the studies. Percentage of mutation of NHL FFPET specimens was determined using an NGS method. The **cobas**® DNA Sample Preparation Kit was used to obtain extracted DNA for the analytical validation studies.

Analytical sensitivity – limit of blank

To assess performance of the **cobas** EZH2 Test in the absence of template and to ensure that a wild-type sample does not generate an analytical signal that might indicate a low concentration of mutation, NHL FFPET EZH2 wild-type specimens were evaluated. Using the analysis prescribed in the CLSI EP17-A2 guideline,[10] the Limit of Blank (LoB) was determined to be zero for replicates when the observed CtR result exceeded the mutant CtR threshold. There was an overall false positive rate of 2.6% (5/195). The discordant specimens were determined to have low levels of mutant not detected by the sequencing. The observed mean LoB CtR for each reaction relative to the clinical cutoffs are provided in Table 4.

Table 4: Observed mean LoB CtR with WT DNA relative to the CtR cutoffs

Targeted Mutation	N	LoB CtR	CtR Cutoff
Y646N	195	20.9	9.0
A692V	195	10.5	6.5
Y646F	195	21.9	8.0
A682G	195	13.8	6.0
Y646X	195	10.7	9.0

Limit of detection using FFPET specimen blends

DNA isolated from 20 NHL FFPET specimens with EZH2 mutations (Y646N, A692V, Y646F, A682G, Y646H, Y646S and Y646C) were blended with DNA isolated from wild-type NHL FFPET samples to achieve 21 unique DNA blends (panels) targeting 10.0%, 7.5%, 5.0%, 2.5%, and 1.0% mutation levels as determined by an NGS method. Twenty-one replicates of each targeted mutation level were tested. The limit of detection for each sample was determined by the lowest percent mutation that gave an EZH2 "Mutation Detected" rate of at least 95% for the targeted mutation, shown in Table 5. The study demonstrates that the **cobas** EZH2 Test can detect mutations in EZH2 exons 16 and 18 with at least 5% mutation level using the standard input of 50 ng DNA per reaction well. The claimed limit of detection for the overall assay is 5% based on the highest LoD achieved (Y646H).

Table 5: Limit of Detection for the cobas EZH2 Test using FFPET sample DNA blends

Exon	COSMIC ID	AA Change	Nucleic Acid Change	Percent Mutation in the Panel Member to achieve ≥ 95% "Mutation Detected" Rate with 50 ng DNA input per reaction well (N=21 Valid replicates)
16	37031	Y646N	TAC>AAC	2.1
				2.6
				2.5
18	220529	A692V	GCA>GTA	2.0
				1.6
				2.7
16	37028	Y646F	TAC>TTC	1.6
				2.2
				1.3
18	220386	A682G	GCA>GGA	1.1
				1.8
				2.0
16	37030	Y646H	TAC>CAC	0.9
				1.9
				4.7
16	37029	Y646S	TAC>TCC	2.3
				3.9
				2.5
16	37032	Y646C	TAC>TGC	0.7
				2.4
				2.9

Limit of detection using cell line blends

Genomic DNA extracted from lymphoma cell lines for EZH2 mutations (Y646N, Y646F, Y646S, Y646C, and A682G) were blended with wild-type cell line DNA to achieve DNA blends (panels). The blends were tested at Neat, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 with an additional dilution of 1:128 for Y646C from a homozygous cell line. A total of 21 replicates of each targeted mutation level were tested using one lot of **cobas** EZH2 Test kit. The final percent mutation was determined by NGS.

The **cobas** EZH2 Test was able to achieve a ≥ 95% detection rate in the EZH2 gene with a percent mutation level ranging from 1.1% to 2.0% with a 50 ng/PCR DNA input, shown in Table 6. The results support the claimed LoD of 5% for the assay.

Table 6: Limit of Detection for the cobas EZH2 Test using cell line DNA

Exon	COSMIC ID	Mutation	Nucleic Acid Change	Percent Mutation in the Panel Member to achieve ≥ 95% "Mutation Detected" Rate with 50 ng DNA input per reaction well (N=21 Valid replicates)
16	37031	Y646N	TAC>AAC	1.9
16	37028	Y646F	TAC>TTC	1.1
18	220386	A682G	GCA>GGA	2.0
16	37029	Y646S	TAC>TCC	1.2
16	37032	Y646C	TAC>TGC	1.7

Method correlation

A total of 104 unique NHL FFPET specimens were tested for the presence of Y646N, Y646F, Y646H, Y646S, Y646C, A682G and A692V mutations with two lots of the **cobas** EZH2 Test Kits and compared with results from NGS to determine the overall percent result agreement between methods.

Table 7 summarizes the positive, negative, and overall percent agreements, with corresponding 95% score confidence intervals, between the **cobas** EZH2 Test and NGS. Overall percent agreement, positive percent agreement and negative percent agreements were ≥ 95% between methods. The comparisons of individual mutations are shown in Table 8 and Table 9.

Table 7: Summary of percent agreement between cobas EZH2 Test and NGS

	Lot 1 vs. NGS	Lot 2 vs. NGS
Positive Percent Agreement	100% (50/50) 95% CI: 92.9%-100%	100% (50/50) 95% CI: 92.9%-100%
Negative Percent Agreement	96.3% (52/54) 95% CI: 87.5%-99.0%	98.1% (53/54) 95% CI: 90.2%-99.7%
Overall Percent Agreement	98.1% (102/104) 95% CI: 93.3%-99.5%	99.0% (103/104) 95% CI: 94.8%-99.8%

Table 8: Per call comparison of the cobas EZH2 Test Lot 1 vs. NGS

		NGS						
		Y646N	**A692V**	**Y646F**	**A682G**	**Y646X**	**WT**	**Total**
cobas EZH2 Test	**Y646N**	13	0	0	0	0	1	14
	A692V	0	4	0	0	0	0	4
	Y646F	0	0	14	0	0	1	15
	A682G	0	0	0	5	0	0	5
	Y646X	0	0	0	0	19	2	21
	WT	0	0	0	0	0	461	461
	Total	13	4	14	5	19	465	520*

*Note: The 520 total results represent 5 different mutation results or a WT result for each of the 104 samples used in the study.

Table 9: Per call comparison of the cobas EZH2 Test Lot 2 vs. NGS

		NGS						
		Y646N	**A692V**	**Y646F**	**A682G**	**Y646X**	**WT**	**Total**
cobas EZH2 Test	**Y646N**	13	0	0	0	0	1	14
	A692V	0	4	0	0	0	0	4
	Y646F	0	0	14	0	0	1	15
	A682G	0	0	0	5	0	0	5
	Y646X	0	0	0	0	19	0	19
	WT	0	0	0	0	0	463	463
	Total	13	4	14	5	19	465	520*

*Note: The 520 total results represent 5 different mutation results or a WT result for each of the 104 samples used in the study.

Cross-reactivity

Sequence homology searches were performed comparing the **cobas** EZH2 Test primer and probe sequences with known sequence databases using BLAST to assess for potential cross- reactivity of non-targeted DNA sequence that may be present in human genomic DNA. Based on sequence similarities, potential cross-reactivity with an EZH2 pseudogene and an EZH2 paralog (EZH1) were evaluated.

To determine the potential cross-reactivity of non-targeted DNA sequences, the cross-reactivity of the **cobas** EZH2 Test was assessed using fifteen specimens consisting of five EZH2 mutant specimens and ten wild-type specimens. Potential cross-reactivity with an EZH2 pseudogene and an EZH2 paralog (EZH1) was evaluated by spiking EZH2 pseudogene and EZH1 plasmid DNA into FFPE genomic DNA from multiple NHL wild-type and EZH2 mutant specimens, at a ratio of approximately 50% plasmid DNA to 50% amplifiable specimen DNA. This study was conducted by one operator using two **cobas z** 480 analyzers and one reagent lot of the **cobas** EZH2 Test.

The **cobas** EZH2 Test exhibited no cross-reactivity when tested with the DNA sequences of non-targeted mutations (EZH2 Pseudogene and EZH2 Paralog).

Interference

Hemoglobin (2 mg/mL, CLSI recommended high concentration[11]) and triglycerides (37 mM, CLSI recommended high concentration[11]) have been shown not to interfere with the **cobas** EZH2 Test when these potential interfering substances were added to the lysis step during the specimen preparation procedure.

Rituximab, Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone were tested at concentrations representing 3 times the peak serum concentration (3X C_{max}) that a drug achieves after its administration or at a concentration equivalent to 3X therapeutic doses distributed in 5 liters of blood. The tested drugs have been shown not to interfere with the **cobas** EZH2 Test when each potential interfering substance was added to the lysis step during the specimen preparation procedure.

Necrotic tissue

NHL FFPET specimens with necrotic tissue content from 0% to 95% do not interfere with the call results of the **cobas** EZH2 Test.

Repeatability

Repeatability of the **cobas** EZH2 Test was assessed using five NHL FFPET EZH2 mutant specimens (Y646N, Y646F, Y646X, A682G, and A692V) and two EZH2 FFPET wild-type specimens. The specimens were tested in duplicate by two operators, using two different reagent lots and two **cobas z** 480 analyzers over eight days. A total of 32 replicates of each specimen were evaluated. The **cobas** EZH2 Test had an overall correct call rate of 100%. Specimens evaluated in the repeatability study had allele frequencies ranging (12% to 26%).

Reproducibility

Reproducibility of the **cobas** EZH2 Test was assessed at three external sites. A 15-member panel of genomic deoxyribonucleic acid (DNA), representing clinically relevant EZH2 mutations and wild-type (WT) DNA was prepared including extraction of DNA from commercially available clinical samples' formalin fixed paraffin embedded tissue (FFPET). Panel members' mutation status was verified externally using a validated sequencing method.
The target level % mutation for panel members were at or near the Limit of Detection (LoD) and at 3x LoD. Panels were provided to each of the 3 testing sites in a blinded fashion. Testing of each panel member was performed in duplicate across 3 lots of reagents over 5 non-consecutive days by 2 operators per site on one instrument per site.

Overall, 91 runs were performed, 90 of which were valid and one invalid. In the 90 valid runs, 2,700 tests were performed, of which 10 tests were invalid (0.4%). All valid test results (2,690) were included in the analyses. Between three lots of reagents, at three sites and six operators, 100% agreement was reached for results from all panel members regardless of mutation level at LoD and 3x LoD.

Table 10 shows the percent agreement with 95% exact CI by panel member from all valid test results. For the WT panel member, 'No Mutation Detected' results were counted as agreement. For the mutant (MT) panel members, 'Mutation Detected' test results were counted as agreement.

Table 10: Overall estimates of agreement by panel member

Panel Member	Number of Valid Tests	Agreement N	Agreement % (95% CI)[a]
Wild-Type	180	180	100 (98.0, 100.0)
Ex16 Y646N LoD	179	179	100 (98.0, 100.0)
Ex16 Y646F LoD	179	179	100 (98.0, 100.0)
Ex16 Y646H LoD	179	179	100 (98.0, 100.0)
Ex16 Y646S LoD	179	179	100 (98.0, 100.0)
Ex16 Y646C LoD	180	180	100 (98.0, 100.0)
Ex18 A682G LoD	180	180	100 (98.0, 100.0)
Ex18 A692V LoD	180	180	100 (98.0, 100.0)
Ex16 Y646N 3x LoD	180	180	100 (98.0, 100.0)
Ex16 Y646F 3x LoD	179	179	100 (98.0, 100.0)
Ex16 Y646H 3x LoD	178	178	100 (97.9, 100.0)
Ex16 Y646S 3x LoD	180	180	100 (98.0, 100.0)
Ex16 Y646C 3x LoD	179	179	100 (98.0, 100.0)
Ex18 A682G 3x LoD	179	179	100 (98.0, 100.0)
Ex18 A692V 3x LoD	179	179	100 (98.0, 100.0)

Note: Results are included as agreement when a valid test of MT panel member has a result of 'Mutation Detected'; or when a valid test of WT panel member has a result of 'No Mutation Detected'.
[a] 95% CI = 95% exact binomial CI.
CI = confidence interval; LoD = limit of detection; MT = mutant; WT = wild-type

The variance component analyses for lot, site/instrument, operator, day and within-run were performed on Ct value for each mutation panel member. Overall, the CV (%) for total imprecision ranged from 1.5% to 1.9% across panel members. Within each component, CV (%) ranged from 0.0% to 1.6% across all panel members.

Lot-to-lot interchangeability

Lot-to-lot interchangeability of various lots of **cobas**® DNA Sample Preparation Kit with various lots of **cobas** EZH2 Test was assessed. Five mutant specimens (Y646N, A692V, Y646F, A682G and Y646S, representing the Y646X group), and four wild type specimens were used for this study. Nine lot combinations were created by crossing three lots of the **cobas**® DNA Sample Preparation Kit with three lots of the **cobas** EZH2 Test. One wild type sample generated a mutation call for the Y646X mutation resulting in the negative percent agreement of 97.2%. A root cause analysis was performed and determined that the unexpected result was sample related and not due to the performance of the reagents. The positive, negative and overall percent agreements across all lot combinations are shown in Table 11.

Table 11: Lot interchangeability percent agreement

	NGS MD	NGS NMD	Total
cobas EZH2 MD	45	1	46
cobas EZH2 NMD	0	35	35
Total	45	36	81
% Agreement	100%	97.2%	98.8%
95% Confidence Interval	92.1% – 100%	85.5%– 99.9%	93.3% – 100%

Clinical performance evaluation

The safety and effectiveness of the **cobas** EZH2 Test for detecting EZH2 mutations in FFPE specimens from patients with relapsed/refractory (R/R) follicular lymphoma (FL) who may benefit from treatment with TAZVERIK™, was established with clinical data generated in the Epizyme Phase 1/2 study E7438-G000-101. The study was an open-label, multi-center study investigating the safety and efficacy of TAZVERIK™ treatment in R/R FL patients with 2 prior systemic therapies, and whose tumor tissue harbored an EZH2 mutation as determined by the **cobas** EZH2 Test.

A total of 258 follicular lymphoma patients were prospectively tested by the **cobas** EZH2 Test using FFPET. The prevalence of EZH2 results with MD (positive) within codon 646, 682, and 692 (Y646F, Y646N, Y646S, Y646H, Y646C, A682G, A692V) was 22.5% (58/258) in FL patients. Of those MD patients, 45 patients were enrolled in the clinical trial, with mutation distribution as shown in Table 12.

Table 12: Proportion of EZH2 mutation distributions by the cobas EZH2 Test

Mutation Status	Count	Proportion (%)	95% CI of Proportion (%)
A682G	4	8.89	(3.51, 20.73)
A682G; Y646X	1	2.22	(0.39, 11.57)
A692V	1	2.22	(0.39, 11.57)
Y646F	12	26.67	(15.96, 41.04)
Y646N	11	24.44	(14.24, 38.67)
Y646N; Y646F	1	2.22	(0.39, 11.57)
Y646X	15	33.33	(21.36, 47.93)
Total	45	100	N/A

Among the 45 patients with EZH2 mutant FL, the median age was 62 years (range 38 to 80), 58% were female and the majority were white. Forty-two out of the 45 FL patients were included in the efficacy analysis. The major efficacy outcome measures were overall response rate (ORR) and duration of response (DOR) according to the International Working Group Non-Hodgkin Lymphoma (IWG-NHL) criteria as assessed by Independent Review Committee. Median duration of follow-up was 22 months (range 3 months to 44 months) for patients with EZH2 mutation positive tumors.

For the FL EZH2 positive patients, 29 out of 42 were considered as responders (5 complete response and 24 partial response), resulting in ORR of 69% with 95% CI of (53%, 82%), as shown in Table 13.

Table 13: Efficacy results for patients with relapsed or refractory Follicular Lymphoma enrolled into EZH2 MD in Study E7438-G000-101

Efficacy Endpoints	TAZVERIK™ EZH2 MD FL Patients
Overall Response Rate (95% CI)*	69% (53%, 82%)
Complete Response	12%
Partial Response	57%
Duration of Response	
Median (95% CI) in months	10.9 (7.2, NE)
Range in months	0.0+, 22.1+

CI: Confidence Interval; NE = not estimable

* Median time to response for patients with EZH2 MD follicular lymphoma was 3.7 months (range 1.6 to 10.9)

Method comparison study to the reference NGS method using samples from the Epizyme Phase 1/2 study E7438-G000-101

The accuracy of the **cobas** EZH2 Test for the detection of mutations within codon 646, 682, and 692 of the EZH2 gene was assessed by comparing **cobas** EZH2 Test results to a validated, next generation sequencing (NGS) test method. A subset of 378 samples representative of the consented patients' population including those diagnosed with the follicular lymphoma screened by the **cobas** EZH2 Test was selected for sequencing. This subset included samples from enrolled and screen failure patients. Out of 378 samples selected, 341 had sufficient DNA or tumor tissue available for sequencing. The comparison of EZH2 results between the **cobas** EZH2 Test and the NGS method is presented in Table 14. The comparisons of individual mutations are shown in

Table 15 for single mutations. Overall the results are consistent with the accuracy data using the procured specimen set as shown in Table 7.

Table 14: Comparison of aggregate results from the cobas EZH2 Test with reference NGS method

		NGS (Reference Method)			
Results		**MD[a]**	**NMD[b]**	**Invalid**	**Total**
cobas EZH2 Test	**MD**	119	3	0	122
	NMD	2	208	3	213
	Invalid	0	3	3	6
	Total	121	214	6	341
Agreement Statistics (Excluding invalids)	**PPA (95% CI)**	98.3% (94.2%, 99.5%)			
	NPA (95% CI)	98.6% (95.9%, 99.5%)			

[a] MD = Mutation Detected; [b] NMD = No Mutation Detected.

Note: All CI (confidence intervals) were calculated by Wilson score method

Table 15: Comparison of the cobas EZH2 Test vs. NGS by for single mutation

	NGS Sequencing						
cobas EZH2 Test	**A682G**	**A692V**	**Y646F**	**Y646N**	**Y646X**	**WT**	**Total**
A682G	5	0	0	0	0	1	6
A692V	0	2	0	0	0	1	3
Y646F	0	0	38	0	0	0	38
Y646N	0	0	0	36	0	0	36
Y646X	0	0	0	0	31	1	32
WT	0	0	1	1	0	208	210
Total	5	2	39	37	31	211	325

For the most up-to-date information regarding TAZVERIK™, refer to the website Drugs@FDA.

Result flags

Explanation of result flags

The source of a flag is indicated in the flag code as outlined in Table 16.

Table 16: Flag source

Flag code starts with	Flag source	Example
M*	Multiple or other reasons	M6
R	Result interpretation	R795
Z*	Analyzer	Z1

* Refer to the **cobas**® 4800 System - User Assistance

All the result flags of the system that are user relevant are listed in Table 17.

Table 17: List of result interpretation flags

Flag Code	Severity	Description	Recommended Action
R700, R706, R712, R718, R724, R730, R736, R742, R748, R754, R760, R766	Error	Mutant Control could not be detected	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that the elbow determination algorithm encountered an error which may occur in the event of an atypical or noisy fluorescence pattern.
R701, R707, R713, R719, R725, R731, R737, R743, R749, R755, R761, R767	Error	Mutant Control could not be detected	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that a negative result occurred for the Mutant Control. Mutant Control DNA may have not been added to one or more wells.
R702, R708, R714, R720, R726, R732, R738, R744, R750, R756, R762, R768	Error	Mutant Control is out of range	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that an observed Ct value for the Mutant Control was above the established threshold (i.e., elbow too high). Possible reasons could be: 1. Incorrect preparation of the working Master Mix. 2. Pipetting error when adding working Master Mix into a well of the microwell plate. 3. Pipetting error when adding Mutant Control into a well of the microwell plate.
R703, R709, R715, R721, R727, R733, R739, R745, R751, R757, R763, R769	Error	Mutant Control is out of range	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that an observed Ct value for the Mutant Control was below the established threshold (i.e., elbow too low). This error may occur in the event of DNA contamination.
R772, R774, R776, R778, R780, R782, R784, R786, R788, R790, R792, R794	Error	Negative Control could not be detected	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that the elbow determination algorithm encountered an error which may occur in the event of an atypical or noisy fluorescence pattern.
R773, R775, R777, R779, R781, R783, R785, R787, R789, R791, R793, R795	Error	Negative Control is out of range	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that a positive result occurred for the Negative Control (i.e., a contamination event occurred).
R796, R801, R806, R816, R821, R826, R836, R841, R846	Error	No target could be detected	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that the elbow determination algorithm encountered an error which may occur in the event of an atypical or noisy fluorescence pattern.
R797, R802, R807, R817, R822, R827, R837, R842, R847	Error	No target could be detected	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that a negative result occurred for the sample (i.e., sample may have not been added to one or more wells).

Flag Code	Severity	Description	Recommended Action
R799, R804, R809, R819, R824, R829, R839, R844, R849	Error	Result is out of range	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that an atypically low Ct value was observed for the sample.
R800, R805, R810, R820, R825, R830, R840, R845, R850	Error	Result is out of range	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that an atypical relationship between the Mutant Ct value and the Internal Control Ct value was observed for the sample.
R811, R831, R851	Error	Internal Control could not be detected	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that the elbow determination algorithm encountered an error which may occur in the event of an atypical or noisy fluorescence pattern.
R812, R832, R852	Error	Internal Control could not be detected	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that the Internal Control result for the sample was not valid. The absence of a valid Internal Control result suggests: 1. Poor quality genomic DNA from the sample. 2. Inadequate sample processing. 3. The presence of PCR inhibitors in the sample. 4. Rare mutations within the regions of the Genomic DNA covered by the Internal Control primers and/or probes. 5. Sample DNA may have not been added to one or more wells. 6. Other factors.
R813, R834, R853	Error	Internal Control out of range	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that the Internal Control result for the sample was not valid. The absence of a valid Internal Control result suggests: 1. Poor quality genomic DNA from the sample. 2. Inadequate sample processing. 3. The presence of PCR inhibitors in the sample. 4. Rare mutations within the regions of the Genomic DNA covered by the Internal Control primers and/or probes. 5. Sample DNA may have not been added to one or more wells. 6. Other factors.
R814, R835, R854	Error	Internal Control out of range	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that an atypically low Internal Control Ct value was observed for the sample. This error may occur if the PCR mixture is overloaded with concentrated genomic DNA.

Additional information

Symbols

The following symbols are used in labeling for Roche PCR diagnostic products.

Table 18: Symbols used in labeling for Roche PCR diagnostic products

Age/DOB	Age or Date of Birth
SW	Ancillary Software
Assigned Range [copies/mL]	Assigned Range (copies/mL)
Assigned Range [IU/mL]	Assigned Range (IU/mL)
EC REP	Authorized representative in the European Community
BARCODE	Barcode Data Sheet
LOT	Batch code
	Biological risks
REF	Catalogue number
Collect Date	Collect date
	Consult instructions for use
Σ	Contains sufficient for <*n*> tests
CONTENT	Contents of kit
CONTROL	Control
	Date of manufacture
D	Distributed by
	Do not re-use
	Female
	For IVD performance evaluation only
GTIN	Global Trade Item Number
IVD	*In vitro* diagnostic medical device
LLR	Lower Limit of Assigned Range
	Male
	Manufacturer
CONTROL –	Negative control
NON STERILE	Non sterile
#	Patient number
?	Patient Name


Peel here

CONTROL + Positive control

QS copies / PCR QS copies per PCR reaction, use the QS copies per PCR reaction in calculation of the results.

QS IU/PCR QS IU per PCR reaction, use the QS International Units (IU) per PCR reaction in calculation of the results.

SN Serial number

Site Site

Procedure Standard Standard Procedure

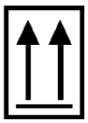
STERILE EO Sterilized using ethylene oxide


Store in the dark


Temperature limit

Test Definition File

CE CE marking of conformity; this device is in conformity with the applicable requirements for CE marking of an *in vitro* diagnostic medical device


This way up

UDI Unique Device Identification

Procedure UltraSensitive Ultrasensitive Procedure

ULR Upper Limit of Assigned Range

Urine Fill Line Urine Fill Line

Rx Only US Only: Federal law restricts this device to sale by or on the order of a physician.


Use-by date


Device for near-patient testing


Device Not for Near Patient Testing


Device for self-testing


Device not for self-testing

Technical support

For technical support (assistance) please reach out to your local affiliate:
https://www.roche.com/about/business/roche_worldwide.htm

Manufacturer and distributors

Table 19: Manufacturer and distributors

Roche Molecular Systems, Inc.
1080 US Highway 202 South
Branchburg, NJ 08876 USA
www.roche.com

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim, Germany

Roche Diagnostics
9115 Hague Road
Indianapolis, IN 46250-0457 USA
(For Technical Assistance call the
toll-free: 1-800-526-1247)

Trademarks and patents

See http://www.roche-diagnostics.us/patents

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