GenVinSet

LACTOSE INTOLERANCE

Instructions for use

Kit for the detection of C13910T y G22018A

Product code GVS-LAC-48 (48 tests)
GVS-LAC-24 (24 tests)

Store from –18 to –30ºC

Rev.03/2021-03-09

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1. Intended use

Genvinset® Lactose Intolerance is a kit for the determination of the polymorphisms C13910T y G22018A of the *MCM6* gene by Real Time PCR using specific TaqMan® probes technology.

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2. Summary and explanation

Lactose is a disaccharide that can be found in mammalian milk and is hydrolyzed in the intestine by the lactase enzyme (also known as lactase-phlorizin hydrolase enzyme or LPH), rendering two absorbable monosaccharides, glucose and galactose. In most children, lactase activity is at its peak during the perinatal period and is essential for nourishment in breast-feeding, but after a few months its activity gradually decreases to almost undetectable levels due to a natural down-regulation of lactase expression (1). However, some humans maintain lactase activity into adulthood, and after 2-12 years there can be found a "lactase non-persistence" group with low lactase activity (primary hypolactasia), and a "lactase persistence" group who sustain the ability to digest milk (2,3). The frequency of this persistence varies greatly depending on the region: it is high in North-European populations (>90% in Sweden and Denmark), whereas it progressively decreases towards the south of Europe and the Middle East (around 50% in France, Spain and some Arabic populations), and is very low in Asian and African populations, although it is common in pastoralist populations (around 1% in China, 5-20% in East Africa) (4).

Low lactase activity during adulthood leads to maldigestion of lactose, which is asymptomatic when no milk or lactose-containing food is ingested (5). However, upon lactose intake and if symptoms appear, lactose intolerance can be diagnosed. Indigested lactose is fermented by colonic microflora in the lumen of the intestine, producing short-chain fatty acids, hydrogen (H2), methane (CH4) and carbon dioxide (CO2) (6). These byproducts can cause abdominal pain and distension, bloating, flatulence and diarrhea (prompted by the acidification of the colon from indigested lactose) (1). Other less common symptoms, such as headache, lethargy, loss of concentration or vertigo appear because the toxic metabolites produced by bacteria can alter cell signaling mechanisms. Symptoms are not normally perceptible until lactase activity is less than 50%. Furthermore, many people with lactase non-persistence can tolerate small amounts of lactose without symptoms, and tolerance can be acquired by adaptation of the intestinal flora with regular lactose intake (although lactase expression does not change) (7). Therefore, not all patients with lactase deficiency develop lactose intolerance. On the other hand, primary hypolactasia should not be mistaken with secondary hypolactasia (caused by conditions such as celiac disease or Crohn's disease) and congenital lactase deficiency, an extremely rare autosomal recessive disease that affects newly-born children and can prove fatal if not treated (8).

The gene encoding lactase (*LCT*) is located on the long arm of chromosome 2. At least six mutations have been associated with lactase expression, but lactases between adults with lactase persistence and hypolactasia are

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identical, since the differences lie in some silent mutations (9). In European populations, a single nucleotide polymorphism (SNP) is responsible for lactase persistence. The variant, known as LCT-13910C>T, is located in intron 13 of the minichromosome maintenance type 6 (*MCM6*) gene, 13,910 bp upstream from the initiation codon of LCT. It is inherited in an autosomal dominant way, with one allele being sufficient to confer lactase-persistence phenotype. On the other hand, LCT-22018G>A polymorphism (located 22,018bp from initiation codon of *LCT*, in intron 9 of *MCM6*) was found in association with LCT-13910C>T in most cases, with some exceptions (2,3,10–12). It is generally accepted that genotyping of LCT-13910C>T polymorphism correlates more accurately with lactase persistence in European populations (where it is ~86%-98% associated) and should therefore be used in the diagnosis of adult-type hypolactasia (13–16). However, this polymorphism is not a good indicator of lactase persistence in non-Europeans, since pastoralist populations of Africa and Asia, although they show a high prevalence of lactase persistence, have a low rate of LCT-13910T allele (17). In Northern China and Japanese Brazilian populations, it was determined that LCT-22018G>A is more accurate for the diagnosis of hypolactasia. (12,18)

Lactase persistence is believed to have appeared in the process of domestication of cattle some 10,000 years ago. The conservation of haplotypes in the alleles involved manifest that these alleles have suffered from a strong positive selection in adult milk-consuming populations (of up to 4-5% per generation), while in non-consumer populations the appearance of lactase persistence is associated to genetic drift. The variety of polymorphisms related to lactase persistence proves mutations developed independently a number of times in different parts around the world (19,20).

There are several ways to diagnose lactose malabsorption and intolerance. A definitive diagnosis is not possible on clinical symptoms alone, since they can be produced by other diseases such as irritable bowel syndrome (IBS) (1). The H2-breath test measures the increase of H2 after lactose intake, yet it can show false positives and more importantly false negatives due to bacterial overgrowth and hydrogen non-producing bacteria in the colon respectively (21). A biopsy from the duodenum which tests for lactase activity is a common form of diagnosis, yet there are some limitations like the non-homogeneous expression of lactase and the invasiveness of the test (1,22). Genetic tests have arisen as a more useful way of detecting lactase persistence, with the limitation of the different SNPs in Arabian and African populations. Other limitations of both the biopsy and genetic tests are that secondary lactase deficiency is not taken into account, and that symptoms are not assessed, whereas they only appear in a fraction of people with lactase deficiency (23).

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3. Procedure principles

The Genvinset® Lactose Intolerance kit allows for the detection of the C13910T and G22018A polymorphisms located in the introns 9 and 13 of the *MCM6* gene by Real Time PCR, monitored with TaqMan® probes. Two wells are required per patient to complete de determination of the two loci.

This technique provides high resolution, high sensitivity, specificity and reproducibility.

(*) See section 12 "Procedure Limitations".

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4. Kit contents

Product code: GVS-LAC-24 (24 tests)

- GVS-CT139-PM: Primer Mix C13910T (PM). 1 vial x 110 µL
- GVS-CT139-MM: Master Mix C13910T (MM). 1 vial x 138 µL
- GVS-GA220-PM: Primer Mix G22018A (PM). 1 vial x 110 µL
- GVS-GA220-MM: Master Mix G22018A (MM). 1 vial x 138 µL
- GVS-LAC-C1: Positive Control 1 (C1). 1 vial x 10 µL
- GVS-LAC-C2: Positive Control 2 (C2). 1 vial x 10 µL
- GVS-RB: Reaction Blank (RB). 1 vial x 100 µL

Product code: GVS-LAC-48 (48 tests)

- GVS-CT139-PM: Primer Mix C13910T (PM). 2 vials x 110 µL
- GVS-CT139-MM: Master Mix C13910T (MM). 2 vials x 138 µL
- GVS-GA220-PM: Primer Mix G22018A (PM). 2 vials x 110 µL
- GVS-GA220-MM: Master Mix G22018A (MM). 2 vials x 138 µL
- GVS-LAC-C1: Positive Control 1 (C1). 1 vial x 10 µL
- GVS-LAC-C2: Positive Control 2 (C2). 1 vial x 10 µL
- GVS-RB: Reaction Blank (RB). 1 vial x 100 µL

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5. Kit storage

In order to ensure a proper performance, reagents should be stored from -18ºC to -30ºC until their expiration date, indicated on the label of the vial. Do not perform more than 3 freeze/thaw cycles to the Primer Mix (GVS-CT139-PM and GVS-GA220-PM) and Master Mix (GVS-CT139-MM and GVS-GA220-MM) vials, as this could decrease the sensitivity of the assay and impair results.

Due to the reagent's photosensitive nature, avoid continuous exposure to light.

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6. Materials required but not supplied

General

- Gloves
- Lab coat

Consumables:

- Filter tips (P200, P100 & P10).
- 1.5 mL Autoclaved tubes.
- q-PCR instrument specific reagents (in the case of using RotorGene Q, only 0.1 ml tubes are allowed).

Equipment

- q-PCR instrument, with FAM and HEX/VIC detection channels. The following devices have been validated:

  - Light Cycler® 96
  - StepOne™
  - DTlite
  - Rotor-Gene Q
  - Applied Biosystems® 7500
  - CFX96™

- Vortex mixer
- Pipettes (P200, P100 & P10)

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7. Sample collection and preparation

This test should only be performed with complete blood samples treated with EDTA anti coagulation agents or citrate. Heparin can interfere with the PCR process and should not be used in this procedure.

The technique is compatible with several DNA extraction methods. Before delivering results with a diagnostic purpose, a validation assay with such extraction method should be done.

Caution

All biological and blood samples should be treated as possibly infectious. When manipulating them, the corresponding basic (universal) precautions should be taken. Samples should always be handled wearing the appropriate personal protection equipment.

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8. Procedures

A) PCR preparation

Precautions

- Thaw all of the kit components before starting the assay, mix and centrifuge them.
- Work on ice or over a cool block.
- The PCR should be setup in the pre-PCR area.
- Use only filtered tips and 1.5 ml autoclaved tubes.
- Use gloves and lab coat at all times.
- In each test performed it is recommended to use one control sample for each C/T or G/A variants.

1. Thaw the samples. Prepare a mix with the Master Mix and the Primer Mix for n+1 samples (one mix for each of the polymorphism (C13910T and G22018A):

	Vol. per sample (µL)
Primer Mix (C13910T or G22018A)	4
Master Mix (C13910T or G22018A)	5

3. Pipette 9 µL of each mix C13910T PM+MM and G22018A PM+MM) into the PCR tubes and add 1µL of DNA or Reaction Blank, in case of the contamination control well.

4. Seal the plate with convenient sealer, and centrifuge 1 min at 360 xg to ensure that all the volume settles to the bottom of the tube.

5. Place the plate in the thermal cycler and start the following program.

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B) Thermal cycler configuration

1. Set up the following amplification program:

	Cycle number	Temperature (ºC)	Time (mm:ss)	Analysis
Denaturation	1	95	05:00	X
Cycle	40	95	00:15	X
		60	1:00	Single
Cooling	1	15	∞	X

2. Set up the reading channels

3. The emitted fluorescence must be read in FAM (495-520 nm) and HEX (535-554 nm) channels. Both fluorescences should be detected in every well (biplex reaction).

NOTE – Special settings for Rotor Gene Q:

a. Open the Rotor-Gene Q – Pure Detection software. Select the tab "Advanced" in the window New Run, and click "New".

b. Select the type of rotor used (only 0.1 mL tubes accepted, see section 6). Select the "Locking Ring Attached" box and continue by clicking "Next".

c. Set the "Reaction Volume" as 10 µl, and identify the operator and the samples.

d. Click on "Edit Profile" and set up the amplification program (see section 8.C.1). Select the step 60 sec at 60 ºC, and click on "Acquiring to Cycling A". Set "Green" and "Yellow" as the fluorescence acquisition channels. Press "OK". Click on "OK" to accept and close the "Edit Profile" window.

e. Click "Gain Optimisation" in the "Run New Wizard" dialog box to open the "Auto-Gain Optimization Setup" window. Scroll down the "Channel Settings" menu and select "Acquiring Channels". Then click on "Add". In the window "Auto-Gain Optimisation Channel Settings", set the following parameters for each channel ("Green" and "Yellow"):

- Tube position = 1
- Target Sample Range: 5 Fl up to 10 Fl
- Acceptable Gain Range: -10 to 10

f. Check the box "Perform Optimisation Before 1st Acquisition", and click on "Close".

g. Select "Next" and then "Start Run" in the "New Run Wizard" window.

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9. Results

Genvinset® Lactose Intolerance constitute a qualitative technique to detect the presence of a C or T polymorphism on the 13910 position of the *LCT* gene, and G or A polymorphism on the 22018 position of the same gene.

It is not necessary to select any passive reference.

In the software that controls the thermocycler, select genotyping as the type of analysis (genotyping, allelic discrimination, scatter plot, etc.) Select the FAM channel (mutation) in the Y axis, and HEX channel (wildtype) in the X axis of the graph.

The genotyping of each sample is determined by calculating the ratio between the detected signals in HEX (normal) and FAM (mutated) channels. Most real time PCR software automatically groups the samples into different regions in a scatter plot. If the axes have been selected as previously described, the dots represented next to the X axis correspond to normal samples (wildype/wild-type), the dots next to the Y axis are homozygous mutated samples, and the group that remains in the middle part of the graph represents heterozygous samples. The contamination control (RB) appears at the coordinates origin.

Genotyping	Amplification in FAM (520nm)	Amplification in HEX (554nm)
Normal (wt/wt)	NO	YES
Heterozygous (wt/mut)	YES	YES
Homozygous mutated (mut/mut)	YES	NO
Reaction Blank	NO	NO

Some instruments need a manual threshold configuration to perform an accurate genotyping assignment. The threshold value for FAM must be set just above the background fluorescence generated by the wildtype control (C1 – positive for HEX). On the other hand, the threshold for HEX must be set just above the background signal generated by the mutated control (C2 – positive for FAM).

The results for the kit are obtained as follows:

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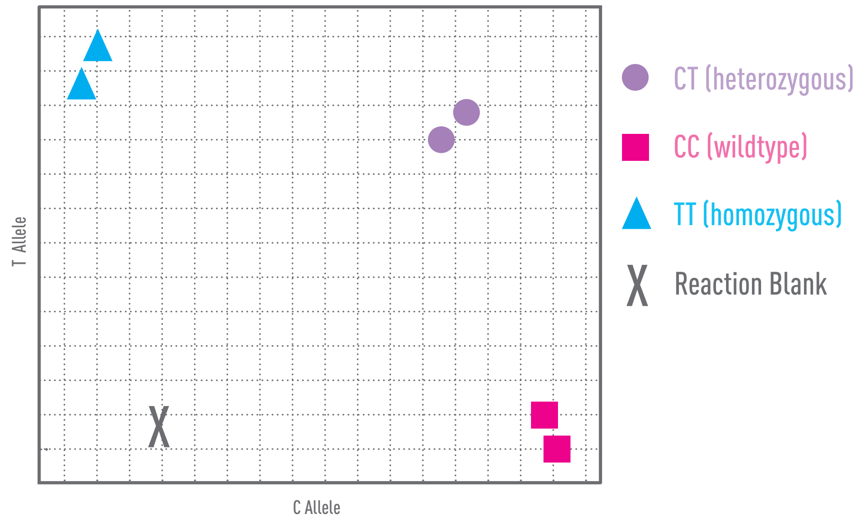
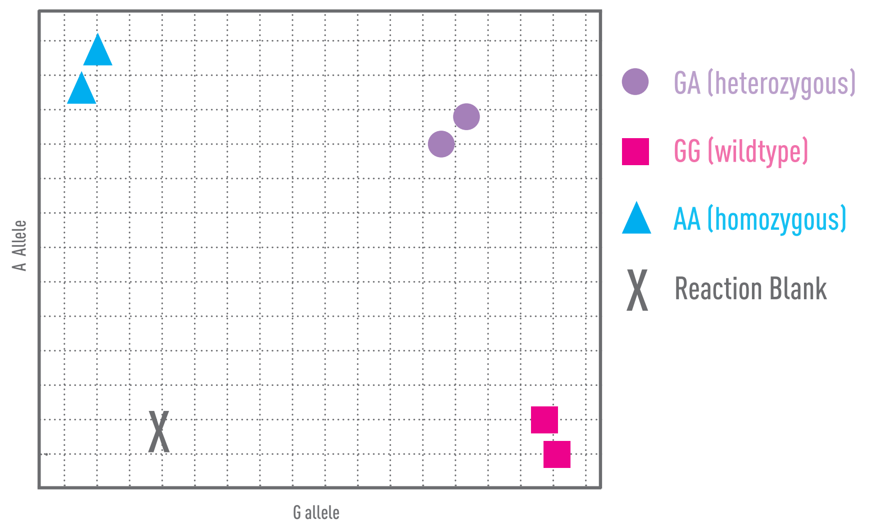
C13910T results

The C allele hybridizes with the HEX-labelled probe, whilst the T allele will pairs with the FAM-labelled probe. When a strong signal in HEX (554nm) is detected and there is no signal or only a background signal in the FAM channel (520nm) the sample is homozygous wildtype (CC). On the contrary, if the only intense signal is that of FAM, the sample is homozygous mutated (TT). Finally, when fluorescence is detected in both FAM and HEX, the sample analysed is heterozygous (CT), since both probes join to amplicons and generate intermediate signals in both channels.



G22018A results

The G allele hybridizes with the HEX-labelled probe, whilst the A allele will pairs with the FAM-labelled probe. When a strong signal in HEX (554nm) is detected and there is no signal or only a background signal in the FAM channel (520nm) the sample is homozygous wildtype (GG). On the contrary, if the only intense signal is that of FAM, the sample is homozygous mutated (AA). Finally, when fluorescence is detected in both FAM and HEX, the sample analysed is heterozygous (GA), since both probes join to amplicons and generate intermediate signals in both channels.



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10. Quality control

Due to the qualitative nature of this test, it will not be necessary to perform a calibration.

The following criteria have to be taken into account in order to validate an assay:

- The Reaction Blank should report negative results in both FAM and VIC/HEX channels. An amplification curve with a Cp>35 value should be considered as negative. A Cp<35 value is associated with a contamination in the Reaction Blank and, therefore, all results from the experiment should be discarded.
- The C1 control sample should produce positive fluorescence only for HEX (wt). The C2 control sample should produce positive results only for FAM (mut).
- DNA samples should always produce a positive control for one of the two channels.
- DNA samples that generate a crossing point (Cp) >35 for HEX and/or for FAM should be considered as doubtful and must be retested performing a new DNA extraction.

The assay must be performed according to the kit recommendations, as well as other quality control procedures that comply with local, federal and/or certifying agencies specifications.

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11. Specific operation data

1. Analytical specificity

The probes align specifically on the regions 13.9kb and 22,0kb of the *LCT* gene. No unspecific alignments have been detected. No cross-reaction phenomena with genomic DNA have been reported.

2. Analytical sensitivity

A dilution assay was performed, using 1:4 serial dilutions of three DNA samples with genotypes CC and GG (normal), CT and GA (heterozygous) and TT and AA (mutated homozygous), obtained by a conventional extraction system, at an initial concentration of 76,90, 93,80 and 100,00 ng/µL, respectively. The following analytical sensitivity results were obtained:

- DNA sample obtained by conventional extraction system: Detection Limit = 1,5 ng/µL (*)

  (*) Cp < 35

3. Diagnostic sensitivity and specificity

In three human genomic DNA studies, 151 samples obtained from several external laboratories were tested. These samples were previously genotyped by another commercial kit based on qPCR or reverse hybridization.

All the tested samples were validated (positive amplification). The following results were obtained:

Genvinset® Lactose Intolerance			
C13910T reaction		G22018A reaction	
CC	51	GG	48
CT	68	GA	73
TT	32	AA	30

There is a 100% match in the results obtained with Genvinset® Lactose Intolerance and the genotyping previously obtained with other commercial kits.

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12. Procedure limitations

- The method detects the C13910T and G22018A polymorphisms of the *MCM6* gene. Mutations or polymorphisms at annealing primer/probe sites are possible and may result of the lack of allele definition.
- All the aforementioned conditions for the setup of the PCR should be carefully controlled. Any performances that do not meet such indications can lead to poor results.
- All procedures described at this document must be done according to general lab best practices and be adjusted to local regulations.
- The q-PCR thermal cycler must be calibrated according to the manufacturers´ recommendations and should be used accordingly to the manufacturer's instructions.
- Do not mix components from other kits or lot numbers.
- Do not use the kit after its expiration date.
- Do not use the kit if there are any suspicions of possible loss of reactivity, contamination, external box deterioration or any other incidence that might affect the kits performance.
- Data and result interpretation should be revised by qualified personnel.
- Discard expired reagents according to applicable regulations.

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13. Troubleshooting guide

Problem

- Probable cause(s)
  - Suggested corrective measure(s)

Reaction Blank (H2O) is positive

- **Primer Mix/Master Mix/Reaction Blank contamination**
  - Repeat the experiment with new Primer Mix/Master Mix/ Reaction Blank aliquots
  - Handle the kit components always according to accepted lab practices in order to avoid contamination
  - Verify manipulation and storage conditions.
  - Discard contaminated reagents
- **Pre-PCR area is contaminated**
  - Confirm that all necessary precautions in the pre-PCR area have been followed
  - Check for possible contamination problems in other PCR techniques
  - Confirm suitability of the used reagents (1.5 ml tubes, pipette tips)
- **Pipetting error**
  - Check that the sample added corresponds to the one indicated on the worksheet

Low or no signal in all samples. Control samples (+/-) are OK

- **Bad quality of DNA samples**
  - Repeat the sample extraction verifying each step (Hemoglobin can interfere with the PCR)
- **Blood processed without previous frozen step**
  - Repeat extraction with a new blood aliquot previously frozen
- **Samples with very low DNA concentration**
  - Check the DNA concentration and repeat the extraction if necessary
- **DNA samples with high concentration**
  - Perform the assay using diluted samples

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Fluorescence intensity too low

- **Kit degradation (Primer Mix and/or Master Mix vials)**
    - Check that the storage of the kit is correct, reviewing both proper temperature conditions and light exposure (which should be avoided)
    - Avoid more than 3 freeze/unfreeze cycles of the Primer Mix vial
    - Aliquote the reagents if necessary
    - Repeat the test with new reagents

C1 control is positive for FAM

- **Cross contamination**
    - Always handle the kit components following all necessary practices to avoid contamination
- **Pipetting error**
    - Check that the sample added corresponds to the one indicated on the worksheet

C2 control is positive for HEX

- **Cross contamination**
    - Always handle the kit components following all necessary practices to avoid contamination
- **Pipetting error**
    - Check that the sample added corresponds to the one indicated on the worksheet

Fluorescence intensity varies

- **The dirtiness on the outside of the tube walls interferes with the signal**
    - Handle all consumables wearing gloves
- **Volume is not settled to the bottom of the well or there are bubbles**
    - Perform a brief centrifugation to ensure that the volume settles to the bottom of the well and to remove all bubbles
- **Pipetting error**
    - Check that the correct volume has been added

There is no fluorescence signal

- **Incorrect reading channels selected**
    - Set the appropriate reading channels

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- **Pipetting error or reagent absence**
  - Control the pipetting and the reaction setup
  - Repeat the PCR
- **No reading channel was selected in the thermal cycler program**
  - Check and modify the thermal cycler program

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15. Notice to purchaser

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16. Changes to version 01

Version	Description of the modification
Rev. 00	First version
Rev. 01	Addition of CE mark and section "Diagnostic sensitivity and specificity"
Rev. 02	Adding CFX96 qPCR instrument to the list of validated thermal-cyclers.
Rev. 03	Section "Results": graphics correction.

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17. Explanation of symbols used on the labels

For in vitro diagnostic use

This product fulfills the requirements of Directive 98/79/EC on in vitro diagnostic medical device

Catalogue number

Lot number

Expiration date

Contents sufficient for <n> tests

Manufactured by

Store at

Keep away from sunlight

Positive control

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