Revised: September 9, 2022

Product Information

HotStart Polymerase Modification Kit

Catalog Number:

29054-T (trial size, sufficient for modifying 0.1 mg polymerase)
29054 (sufficient for modifying 0.5 mg polymerase)

Kit Contents

Component	29054-T	29054
A: Reaction Buffer	29054-TA, 50 uL	29054-A, 75 uL
B: Modifying Reagent	29054-TB, 10 uL	29054-B, 50 uL
C: Storage Buffer	29054-TC, 200 uL	29054-C, 650 uL
Microcentrifuge Ultrafiltration Vial, 10K MWCO (99956)	1 each	1 each
Lumitein™ Protein Gel Stain, 100X (21002)	2 mL	2 mL

Storage and Handling

Store desiccated at -20°C. Kit is stable for at least 6 months from date of receipt when stored as directed.

Lumitein™ 100X and Lumitein™ 1X staining solution can be stored at room temperature or 4°C, protected from light. Lumitein™ 100X is stable for at least 1 year from date of receipt when stored as directed.

Product Description

The HotStart Polymerase Modification Kit provides an easy way to reversibly modify lysine residues of thermostable DNA polymerase, rendering the enzyme inactive. Hot start modification of DNA polymerase for PCR prevents amplification of non-specific PCR products due to low stringency annealing of primers at low temperature during reaction assembly. Following this protocol, activity at room temperature should be eliminated for modified polymerases, reducing the potential of primer-dimers and non-specific amplification. The modification is reversed after heating to 95°C and the polymerase modified using this protocol should have its activity effectively restored to 100% after 2 minutes at 95°C under PCR conditions.

The polymerase modification reaction is very robust and can reach 90% completion within one hour. The degree of polymerase modification can be verified by non-denaturing polyacrylamide gel electrophoresis (PAGE) using Biotium's highly sensitive Lumitein™ red luminescent protein gel stain (included), by using a colorimetric protein stain such as One-Step Blue® (Cat. No. 21003, not provided), or by using the EvaEZ™ Polymerase Activity Kit (Cat. No. 29051, sold separately).

Experimental Protocols

Before you begin

- Dilute or concentrate the polymerase to 1 mg/mL for optimal labeling. If necessary, perform Protocol 1 to concentrate the polymerase by ultrafiltration.
- Free amines and DTT will interfere with the modification reaction. Glycerol should be kept under 5% and Tris under 100 mM. If necessary, perform Protocol 1 to remove interfering substances by ultrafiltration.
- If the polymerase does not require concentration and no interfering substances are present, proceed to Protocol 2.

Materials required but not provided

- 1X PBS
- Non-denaturing Tris-glycine PAGE mini-gel (8% or 4-12% polyacrylamide)
- Non-denaturing sample buffer
- 0.05% SDS/7.5% acetic acid in water for Lumitein™ gel staining
- Methanol and acetic acid for Lumitein™ gel staining

1. Purification and concentration of polymerase using ultrafiltration

Notes:

a. Ultrafiltration is only required if the polymerase contains interfering substances. See "Before you begin" for details.

b. Additional 10K MWCO Ultrafiltration Vials can be purchased separately (Cat. No. 22004).

c. Caution: Avoid touching the membrane of the filtration vial with the pipette tip during liquid transfer. Any damage to the membrane may result in loss of polymerase. You may wish to save the filtrate until verification of sample recovery.

1.1 Load the polymerase solution (0.3 mL maximum) into the sample reservoir of the microcentrifuge ultrafiltration vial (Cat. No. 99956) and centrifuge at 14,000 x g for a few minutes until nearly all of the liquid is in the lower collection tube. Filtration rate will depend on volume, buffer composition, and protein concentration.

1.2 Discard filtrate and add additional polymerase solution to the upper chamber (0.3 mL maximum). Repeat until all of the polymerase solution has been filtered.

1.3 To concentrate polymerase without removing interfering substances, proceed to step 1.5; to perform buffer exchange, proceed to next step.

1.4 Dilute the concentrated polymerase in the upper chamber to ~0.3 mL with 1X PBS and centrifuge. We recommend performing at least two cycles of buffer exchange. Additional cycles will increase purity but decrease total yield.

1.5 Add 1X PBS to the upper chamber to obtain a final concentration of 1 mg/mL polymerase. Carefully pipette up and down to suspend the polymerase in the buffer. Transfer the polymerase solution to a clean microcentrifuge tube.

2. Chemical modification of polymerase

2.1 Warm the Modifying Reagent and Reaction Buffer to room temperature and vortex to mix well before use. **IMPORTANT:** Briefly centrifuge vials to collect all liquid at the bottom of the vial before opening. Examine Reaction Buffer for signs of precipitation; if white precipitate is visible, gently warm to 35°C and vortex Reaction Buffer to fully dissolve, permit to cool back to room temperature before use.

2.2 Reserve some unmodified polymerase for verification steps (see Protocols 3 and 4).

2.3 Add 1/10 volume of Reaction Buffer (component A) to the polymerase solution, mix thoroughly. Add 1/10 volume of Modifying Agent (component B) to the polymerase solution, mix quickly. Gently rock or shake the reaction mixture at room temperature for 1 hour.

Notes:

a. You may leave the reaction overnight on a mixer at 4°C to allow the reaction to approach 100% completion. A speed and temperature control mixer (e.g., Eppendorf Thermomixer®) is ideal to maintain temperature at 4°C with shaking under 300 rpm.

b. Small white precipitates may be observed at the beginning of the reaction, but the solution should be clear after 2 hours.

2.4 Briefly centrifuge the reaction solution; save the supernatant and discard any precipitate.

2.5 Transfer the supernatant containing modified polymerase to a clean tube and add an equal volume of Storage Buffer (component C). Set aside 5-20 uL for verification of modification and store the remaining modified polymerase at -20°C.

Note: If modifying less than 0.5 mg polymerase per reaction, store unused HotStart Polymerase Modification Kit reagents at -20°C for future use.

3. Verify polymerase modification by PAGE and Lumitein™ staining

3.1 Run unmodified and modified polymerase on a non-denaturing Tris-glycine PAGE mini-gel (8% or 4-12% polyacrylamide). Load 200-500 ng of polymerase per lane. Use a non-denaturing sample buffer and do not heat samples before electrophoresis.

Note: A colorimetric protein stain such as One-Step Blue® (Cat. No. 21003, not provided) can be used instead of Lumitein™ staining. For colorimetric staining we recommend increasing the amount of polymerase loaded to 1-3 ug protein per lane.

3.2 After electrophoresis, place the gel into a clean gel staining container containing 0.05% SDS/7.5% acetic acid and soak for 30 minutes with gentle rocking. Use enough solution so that the gel is completely submerged and floating freely (~100 mL for one mini-gel).

3.3 Prepare Lumitein™ 1X staining solution by combining 116 mL of water, 56 mL methanol, 26 mL acetic acid, and 2 mL Lumitein™ 100X (Cat. No. 21002).

3.4 Decant the SDS/acetic acid soaking solution from the staining container and add at least 80 mL of Lumitein™ 1X staining solution per 8 x 8 cm mini-gel. Using an insufficient volume of staining solution may result in low signal.

Note: Additional Lumitein™ protein gel stain can be purchased separately (see Related Products).

3.5 For the best sensitivity, stain the gel for 90 minutes with rocking, protected from light. For rapid results, stain for 30 minutes with rocking, protected from light.

3.6 Destaining may not be required, but may reduce background. Decant Lumitein™ 1X staining solution and wash the gel in 100 mL of 30% methanol/15% acetic acid/55% water with rocking for 5 min. Decant the destaining solution, add at least 100 mL of deionized water and rock for at least another 5 minutes before viewing/imaging. Alternatively, destaining and rinsing can be accomplished in a single step by washing the gel in 100 mL of deionized water for 20 minutes with rocking.

3.7 Image the gel using a standard 300 nm UV transilluminator with an ethidium bromide emission filter.

Note: Lumitein™ can also be imaged using a blue light transilluminator or fluorescence laser scanner with 488 nm or 532 nm laser excitation and a detection window centered around 610 nm emission (such as the SYPRO® Ruby channel). Using 532 nm excitation may give lower background fluorescence compared to 488 nm excitation.

3.8 Modified polymerase will migrate faster compared to unmodified polymerase. A representative gel showing the migration shift of polymerase after modification is shown in Figure 1.

Note: Some unmodified protein may not enter the lane because it is neutral or positively charged in the electrophoresis buffer.

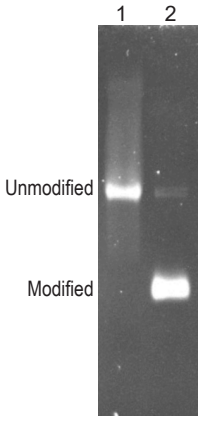

Figure 1. Lumitein™-stained 4-12% Tris-glycine non-denaturing PAGE gel showing the typical mobility shift of Taq polymerase before (1) and after (2) modification.

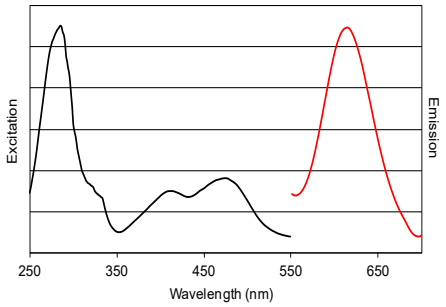

Figure 2. Excitation and emission spectra of Lumitein™.

4. Optional: Verify polymerase modification using EvaEZ™ Fluorometric Polymerase Activity Assay Kit (Cat. No. 29051, not provided)

Note: For more detailed information, protocols, and troubleshooting advice, please see the EvaEZ™ Fluorometric Polymerase Activity Assay Kit product page at biotium.com.

4.1 Label four PCR tubes as A, B, C and D.

4.2 Assemble the following reaction for modified polymerase in Tube A on ice:

Reaction component	Amount required per 20 uL reaction
2X EvaEZ™ Polymerase Activity Mix	10 uL
Modified polymerase (equivalent to 0.5 U/uL)	1 uL
H_2O	9 uL

4.3 Adjust concentration of modified polymerase to ~5 U/uL. Assemble the following in Tube B. This sample will be heated to activate the enzyme:

Reaction component	Amount required per 20 uL reaction
2X EvaEZ™ Polymerase Activity Mix	10 uL
Modified polymerase (equivalent to 5 U/uL)	1 uL
H_2O	9 uL

4.4 Heat Tube B at 95°C for 10 minutes, and place on ice.

Notes:

a. Under typical PCR conditions, HotStart modification of Taq is completely reversed after 2 minutes of heat activation. However, longer activation time may be required for different enzymes, or if the polymerase is at a higher concentration. Therefore, we recommend heating for 10 minutes to ensure complete HotStart reversal for confirmation of activity recovery. Other heat activation time points could be included if desired.

b. Other buffers may be used to dilute polymerase for heat activation, but buffer components such as glycerol may slow reversal of HotStart modification.

4.5 Assemble the following reaction in Tube C on ice:

Reaction component	Amount required per 20 uL reaction
2X EvaEZ™ Polymerase Activity Mix	10 uL
Reaction from Tube B	2 uL
H_2O	8 uL

4.6 Assemble the following reaction in Tube D on ice:

Reaction component	Amount required per 20 uL reaction
2X EvaEZ™ Polymerase Activity Mix	10 uL
Unmodified polymerase from step 2.2 (0.5 U/uL)	1 uL
H_2O	9 uL

4.7 Place Tube A, C, and D at 25°C and monitor green fluorescence on a real time thermocycler. Reactions can also be carried out in a fluorometer cuvette or a plate reader where the temperature can be accurately controlled.

Notes:

a. Hot start modification may not completely inactivate polymerase activity, but 90-99% inactivation can result in significant enhancement of performance.

b. Heating may not result in recovery of 100% of polymerase activity.

Example EvaEZ™ assay data for Taq Polymerase at 25°C is shown in Figure 3. Tube A did not show activity, while Tubes C and D showed activity as measured by an increase in fluorescence, demonstrating that modification inactivated the polymerase and was reversed upon heating.

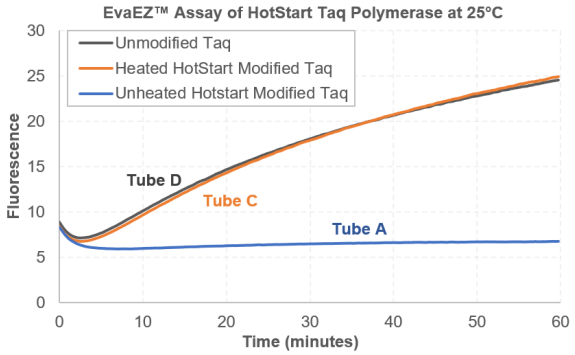

Figure 3. EvaEZ™ Polymerase Activity assay showing Taq polymerase activity at 25°C without modification (Tube D), after modification (Tube A), and after reactivation of the modified enzyme by heating (Tube C).

Related Products

Cat. No.	Product
22004	Ultrafiltration vial, 10K MWCO (pack of 5)
21001	Ultrafiltration vial, 3K MWCO (pack of 5)
21002	Lumitein™ Protein Gel Stain, 100X
21003	One-Step Blue® Protein Gel Stain
21004	One-Step Lumitein™ Protein Gel Stain
21005	One-Step Lumitein™ UV Protein Gel Stain
29051	EvaEZ™ Polymerase Activity Kit
29050	Cheetah™ Hotstart Taq DNA polymerase
29053	WarmStart™ Modification Kit
29087	VeriFluor™ Far-Red Passive Reference Dye, 400X in Water
31000	EvaGreen® Dye, 20X in H_2O
31042	Forget-Me-Not™ EvaGreen® qPCR Master Mix (2-Color Tracking)
31045	Forget-Me-Not™ EvaGreen® qPCR Master Mix (Low ROX)
31046	Forget-Me-Not™ EvaGreen® qPCR Master Mix (High ROX)
31044	Forget-Me-Not™ Universal Probe qPCR Master Mix
31077	EvaGreen® Plus Dye, 20X in Water

Please visit our website at www.biotium.com for information on our life science research products, including environmentally friendly EvaGreen® qPCR master mixes, Cheetah Hotstart Taq DNA polymerase, fluorescent CF® Dye antibody conjugates and reactive dyes, apoptosis reagents, fluorescent probes, and kits for cell biology research.