

Preservative efficacy study for Benzalkonium Chloride solutions: determining the minimal concentration using AET

Antimicrobial effectiveness test reveals the lowest effective concentration of Benzalkonium Chloride needed to maintain antimicrobial efficacy in pharmaceutical formulations.

Benzalkonium Chloride (BKC or BAK/BAC/BZK) is a powerful preservative used to prevent microbial contamination in ophthalmic solutions, nasal sprays, oral, and topical preparations. While effective at low concentrations, reducing BKC is important to minimize irritation risks. This study identified the lowest effective concentration of BKC through Antimicrobial Effectiveness Testing (AET) and developed an HPLC-based method to verify the concentration of tested solutions. Filter compatibility tests were also performed to ensure consistent BKC potency. These findings offer key insights for analytical and formulation scientists aiming to optimize BKC use while balancing product efficacy and patient safety.

Benzalkonium chloride: a trusted preservative for ophthalmics and beyond

BKC, known for its potent antimicrobial properties, has long been the preservative of choice in ophthalmic and nasal formulations¹⁻³. Its broad-spectrum efficacy against bacteria, fungi, and viruses makes it essential in maintaining the sterility of multi-dose products, especially ophthalmic solutions where contamination risks can lead to severe eye infections.

Beyond ophthalmics, BKC is widely used in nasal sprays, oral formulations, and topical preparations, demonstrating its versatility in pharmaceutical applications. Its ability to provide antimicrobial protection at low concentrations is a key advantage, helping balance efficacy with patient comfort.

BKC's preservative effectiveness stems from its unique chemical structure. As a quaternary ammonium compound, BKC consists of a benzyl group, an alkyl chain, and a permanently charged quaternary ammonium ion (*Figure 1*). In pharmaceutical formulations, BKC typically contains a mixture of homologs, primarily C12 and C14 alkyl chains, which influence its surfactant properties and antimicrobial activity. Understanding this homolog distribution is critical for ensuring consistent performance across formulations.

BKC exerts its antimicrobial action by disrupting microbial cell membranes, destabilizing the lipid bilayer, and causing leakage, which ultimately leads to cell death⁴ (*Figure 2*). This mechanism, along with its broad-spectrum efficacy, makes BKC a reliable preservative across a range of pharmaceutical products.

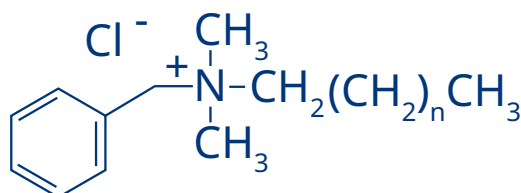


Figure 1: Chemical structure of BKC (CAS 8001-54-5).

C12 homologue: n = 10, MW 339.99, and CAS 139-07-1

C14 homologue: n = 12, MW 368.05, and CAS 139-08-2

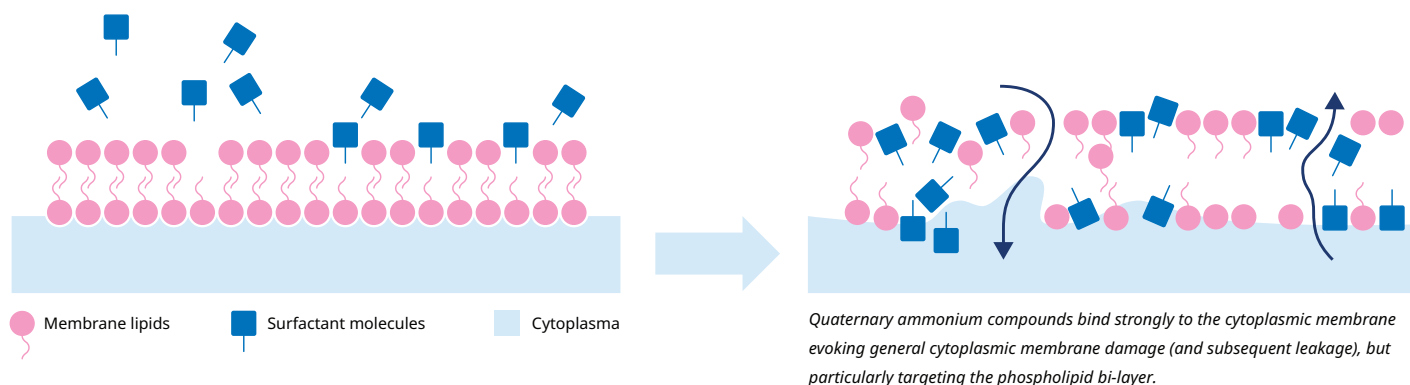


Figure 2: BKC disrupts microbial cell membranes by interacting with the lipid bilayer, causing membrane destabilization and leakage, which leads to cell death.

Striking the balance: optimizing BKC in ophthalmic formulations

The use of BKC in ophthalmic formulations requires a careful balance between its benefits and potential drawbacks. Its broad-spectrum antimicrobial activity ensures robust protection, even at low concentrations, making it essential for maintaining the sterility of multi-dose ophthalmic products. Without preservatives, these products are highly susceptible to contamination, which can lead to serious infections⁵.

However, BKC's effectiveness can pose challenges with long-term or frequent use. It is known to cause irritation in some patients, especially with repeated applications⁶. Therefore, it is crucial to find the right balance—using a concentration high enough to provide effective antimicrobial protection while minimizing the risk of irritation.

Finding the optimal concentration: determining the lowest effective BKC dose

While BKC's antimicrobial strength is well-documented, finding the lowest effective concentration that balances efficacy with minimal irritation remains a challenge. To complement existing Minimum Inhibitory Concentration (MIC) studies⁷, an investigation was conducted to determine the minimum BKC concentration needed for robust antimicrobial protection of a finished drug formulation.

Following USP and EU Pharmacopoeia guidelines for Antimicrobial Effectiveness Test (AET) in ophthalmic products⁸, BKC concentrations ranging from 5 ppm to 100 ppm were tested in a formulation mimicking a basic eye drop solution. The results confirm BKC's strong antimicrobial properties while providing valuable insights for analytical and formulation scientists looking to optimize preservative levels, ensuring both efficacy and patient comfort.

High-performance liquid chromatography (HPLC) analysis of BKC

To ensure the correct concentration and homolog distribution of BKC samples before conducting the AETs, a robust HPLC method was implemented, which is commonly used for analyzing BKC homologs in various formulations⁹. Using the BKC USP Reference Standard (RS) from Millipore Sigma (USP cat. no.: 1050993, USP Lot No.: R121K0, CAS No.: 8001-54-5), the presence of the C12 and C14 homologs was verified and confirmed the validity of the preparation methods.

Early tests revealed that BKC adsorbed onto regular HPLC glass vials (Fisher Scientific cat. no. 03-391-15), leading to potential losses. To prevent this, a switch to Shimadzu vials, minimized sorption loss and improved measurement accuracy.

BKC solutions were prepared from FeF BKC Solution 50% (Novo Nordisk Pharmatech) at concentrations of 5, 10, 25, 50, and 100 µg/mL. Since the BKC product contained only the C12 and C14 homologs, the total area of these peaks was used for potency calculations. Table 1 summarizes the HPLC setup.

Table 1: HPLC method for BKC analysis

HPLC System	Shimadzu 2050C-3D
Column	Phenomenex Luna, 150×4.6 mm, 5 µm CN, 100 Å (Cat#00F-4255-E0)
Column Temp	40°C
Mobile Phase (isocratic)	60% Channel A: Water with 0.085% v/v Phosphoric acid (pH 2.3) 40% Channel B: Acetonitrile
Flow Rate	1 mL/min
Run time	15 min
UV Detection	214 nm; total area of homologs used for potency calculations
HPLC vial	Shimadzu glass vials, Type I borosilicate, Part No. 220-97331-30, Lot No. 8000047033/330824202212999

The method proved effective, meeting system suitability with homolog resolution greater than 2, peak area RSD under 2.0%, and a tailing factor below 2. Linearity between UV response and BKC concentration was excellent ($R^2 > 0.99$) (Figure 3).

When comparing C12 and C14 retention times from FeF BKC to the USP RS, the total peak area was approximately 91% of

the USP RS. This discrepancy was attributed to differences in homolog distribution, as the USP RS is labeled for qualitative use. Therefore, the assay value from the FeF BKC batch COA (49.2% w/v or 50.2% w/w) was used for all calculations.

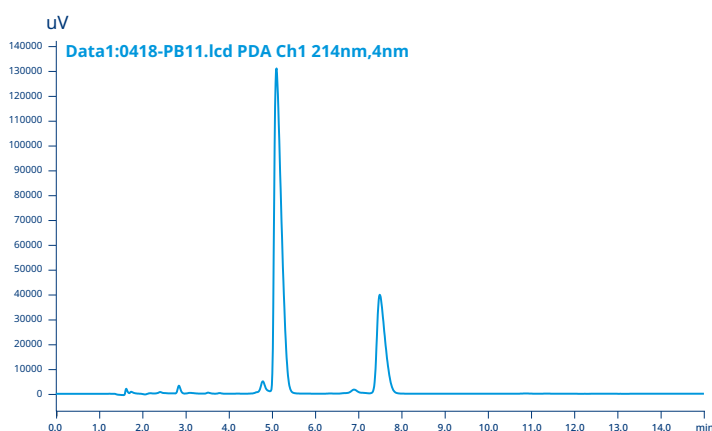
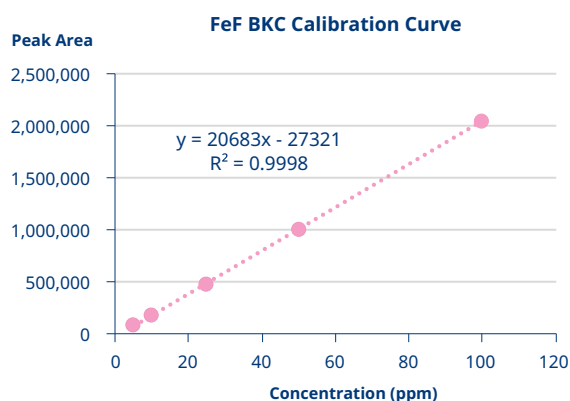
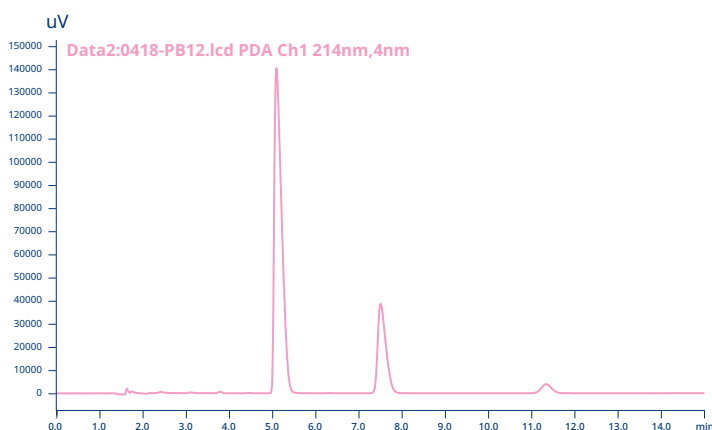


Figure 3: Left: Representative chromatograms of ~100 µg/mL BKC prepared from USP RS (pink) and FeF BKC (blue line), with a typical calibration curve (right) from FeF BKC standard solutions.

Filter compatibility: preserving BKC potency during sterile filtration

One challenge in formulating with BKC is its tendency to adsorb onto filtration media during sterile filtration¹⁰. This can result in significant BKC loss, especially in low-concentration solutions, and compromise the preservative efficacy in final products. To address this, the compatibility of BKC was tested with two types of vacuum filter membranes:

- Polyether Sulfone (PES): 0.2 µm pore size, 37 mm diameter (Genesee Scientific, 25-227)
- Nylon: 0.2 µm pore size, 50 mm diameter (Thermo Nalgene, 0974024A)

Filtered BKC solutions were collected in fractions and analyzed via HPLC, with pre-filtration samples used as controls. Initial tests showed significant BKC loss in the early filtrate fractions, but recovery improved as more solution passed through and the filters became saturated (*Table 2*). Nylon filters exhibited less BKC adsorption than PES filters, making them more suitable when maintaining BKC concentration is critical.

Further testing showed that BKC adsorption was concentration-dependent and saturable. Pre-saturating the Nylon filters with 200 mL of BKC solution ensured consistent recovery in the subsequent filtrate collection (*Tables 3 and 4*). This pre-saturation step was applied to all five BKC solutions prepared for the AET study.

Table 2: Recovery of 5 µg/mL BKC solutions (USP RS) after filtration through PES and Nylon vacuum filters. Nylon filters showed less adsorption than PES filters

Filtrate Fraction # (20 mL each)	% Recovery PES Filter	% Recovery Nylon Filter
Pre-filtration control	100	100
1	8	30
2	72	75
3	85	89
4	92	92
5	93	95

Table 3: Recovery of 5 and 100 µg/mL FeF BKC solutions after filtration through Nylon vacuum filters

Filtrate Fraction # (50 mL each)	% Recovery 5 µg/mL BKC	% Recovery 100 µg/mL BKC
Pre-filtration control	100	100
1	66	89
2	96	99
3	98	100

Table 4: Recovery of 5 µg/mL FeF BKC solutions after filtration through Nylon vacuum filters

Filtrate Fraction # 50 mL each)	% Recovery 5 µg/mL BKC
Pre-filtration control	100
1 & 2 combined	78
3	98
4	98
5	98

These results can help guide analytical scientists regarding the selection of appropriate filters and pre-filtration volumes to ensure the stability and efficacy of BKC in their products.

Benchmarking Benzalkonium Chloride: a comprehensive antimicrobial efficacy test across concentrations

To determine the lowest effective concentration of BKC that meets stringent antimicrobial efficacy standards, a comprehensive AET following USP <51>¹¹ and Ph. Eur. 5.1.3¹² guidelines was conducted. These tests assessed BKC's performance at concentrations of 5, 10, 25, 50, and 100 ppm against a variety of microorganisms commonly associated with contamination in ophthalmic and nasal products:

- *Staphylococcus aureus* (ATCC 6538): Gram-positive bacteria
- *Pseudomonas aeruginosa* (ATCC 9027): Gram-negative bacteria

- *Escherichia coli* (ATCC 8739): Gram-negative bacteria
- *Candida albicans* (ATCC 10231): Yeast
- *Aspergillus brasiliensis* (ATCC 16404): Mold

BKC solutions were prepared in a buffer solution (30 mM pH 6.5 sodium phosphate with added glycerin for an osmolality of 275 mOsm/L) and filtered through sterile Nylon vacuum filters under aseptic conditions. To minimize loss from adsorption, the 200 mL pre-saturation volume identified in the filter compatibility tests was used. Each solution was tested to confirm pH, osmolality, and potency.

At designated timepoints, aliquots were removed, and the BKC was neutralized using Dey-Engley (D/E) neutralizing broth. The remaining microbial population was then assessed. LUMIFY, an over-the-counter ophthalmic product containing 100 ppm BKC, served as a positive control to validate experimental conditions and provide a reliable benchmark.

Table 5: Acceptance criteria according to USP <51> for category 1 products and Ph. Eur. 5.1.3 for parenteral and ophthalmic preparations

	USP <51>	Ph. Eur. 5.1.3
Bacteria	Reduction of at least 1 log by Day 7 and at least 3 log by Day 14, with no increase over next 14d	Criteria A: Reduction of 2 log after 6h, 3 log after 24h, no increase at 28d
		Criteria B: Reduction of 3 log after 14d, no increase over next 14d
Yeast and Molds	No increase in counts at Days 7, 14, or 28, with "no increase" defined as no more than a 0.5 log ₁₀ rise compared to previous counts	Criteria A: Reduction of 2 log after 14d, no increase over next 14d
		Criteria B: Reduction of 1 log after 14d, no increase over next 14d

The AET results (Figure 4) demonstrate the antimicrobial effectiveness of BKC across different concentrations. While the 5 ppm solution failed to meet the acceptance criteria outlined in Table 5, concentrations of 10 ppm and above effectively reduced microbial populations in compliance with both USP and EP guidelines.

Similar results were obtained by Asada et al. in their study of anti-glaucoma solutions containing only the C12 homologue of BKC (BKC-12), following Japanese pharmacopoeia (JP) standards¹³. Their tests, published in Japanese with an abstract available in English, showed that ophthalmic solutions with more than 0.0005% (5 ppm) BKC-12 met the JP preservative efficacy criteria, further supporting the robustness of BKC.

Although the AET is a pass/fail test, a clear trend is visible in the results: as the BKC concentration increases, the onset of antimicrobial activity accelerates, indicating a concentration-dependent response where higher levels lead to faster microbial reduction. For example, while the 10 ppm BKC met the minimum acceptance criteria for *A. brasiliensis*, the higher concentrations led to a faster and more significant reduction of the populations. This is particularly critical during the early phase, such as the first 7 days post-manufacture, when the risk of contamination is highest.

Thus, although 10 ppm BKC meets regulatory requirements, adopting higher concentrations, such as 25 ppm or greater, could enhance product stability and safety, ensuring robust protection during these critical early stages.

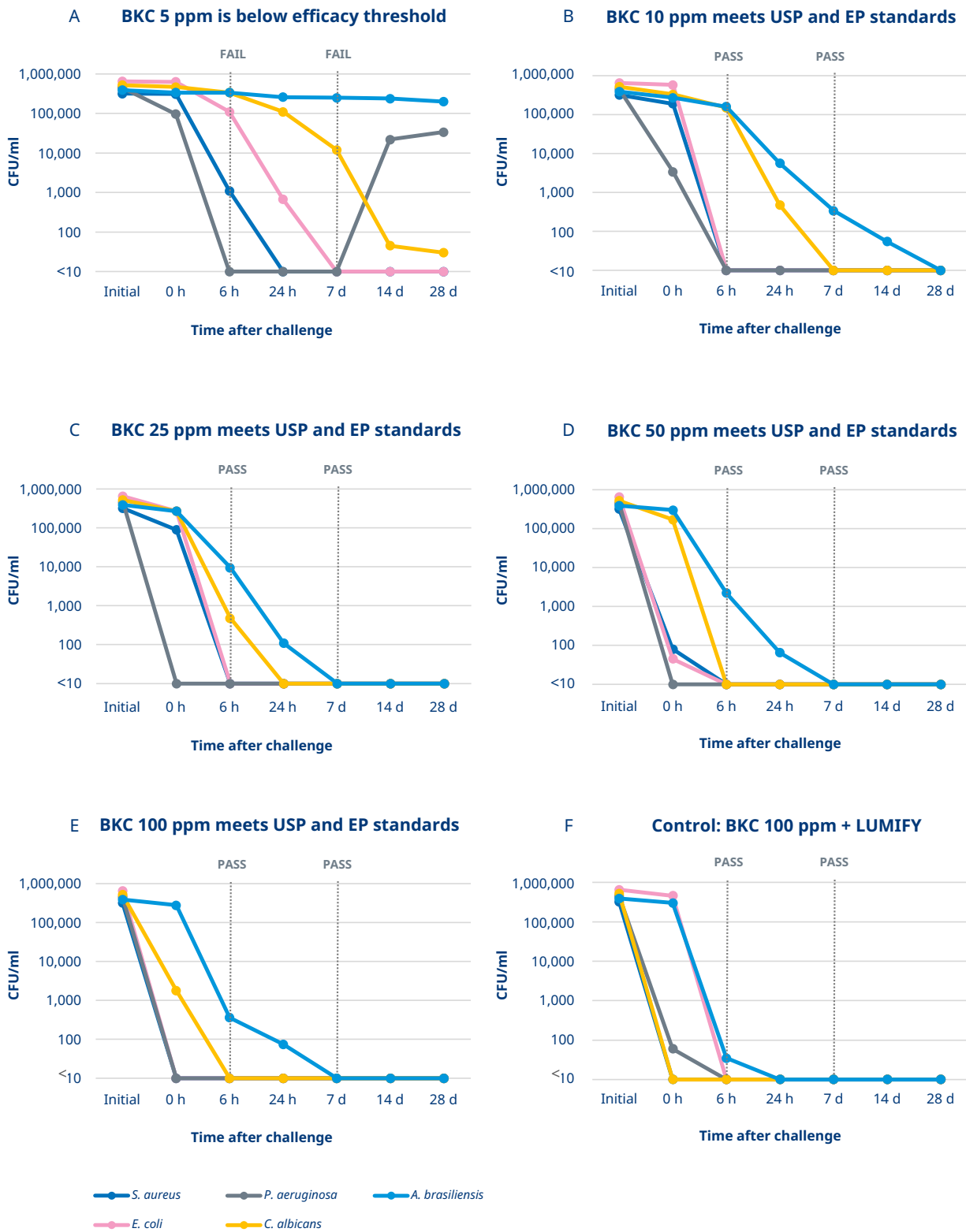


Figure 4: Visualizing BKC antimicrobial activity. All BKC concentrations, except for the 5 ppm BKC met the USP and Ph. Eur. (EP) acceptance criteria. Higher BKC concentrations reduced the microbial populations more quickly.

Key takeaways for formulation and analytical considerations

These findings highlight the necessity of careful selection when determining BKC levels to ensure patient safety and product efficacy. The data underscores the importance of robust analytical techniques like HPLC and optimal filtration processes in measuring preservative potency and overall product quality. Key takeaways from this study include:

- BKC concentrations of 10 ppm and above meet the antimicrobial efficacy standards required by USP <51> and Ph. Eur. 5.1.3.
- Very low concentrations, such as 5 ppm, fail to achieve the necessary antimicrobial protection.
- Effective preservation can be achieved while minimizing irritation by selecting the optimal BKC concentration for sensitive applications like ophthalmics.
- HPLC techniques are crucial for verifying the concentration and homolog distribution of BKC in formulations. They must be carefully validated to ensure accurate recovery and potency measurements.
- Filter selection and pre-saturation strategies help preserve BKC concentration during filtration, ensuring consistent potency in the final product.

Novo Nordisk Pharmatech's Benzalkonium Chloride: setting the standard for quality and efficacy

Sourcing high-quality ingredients like BKC from a cGMP-certified supplier is a fundamental step in ensuring product safety and consistency. At Novo Nordisk Pharmatech, we adhere to the highest quality standards, providing pharmaceutical-grade BKC that meets stringent regulatory requirements.

By partnering with a trusted cGMP supplier, manufacturers can reduce variability, ensure batch-to-batch consistency, and minimize the risk of contamination. This helps safeguard both the manufacturing process and the end product, ensuring that patients receive safe, reliable treatments. At Novo Nordisk Pharmatech, we are committed to supporting the pharmaceutical value chain with products that offer the highest standards of purity, consistency, and safety.



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