Article

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Optimizing Primary Antibody Concentration to Improve BOND-III Immunohistochemistry Staining Quality

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Background: With the advancement of technology, fully automated immunohistochemistry staining systems have been widely adopted in clinical pathological diagnosis due to their standardized operation and high reproducibility. Nevertheless, the specificity and accuracy of staining outcomes are critically dependent on the concentration of the primary antibody. Therefore, the systematic optimization of primary antibody concentration to improve immunohistochemistry staining quality remains a key issue to be addressed. This study aims to optimize the primary antibody concentration for the BOND-III platform to establish standardized protocols and enhance the reliability of immunohistochemistry staining results.
Methods: This study was performed using pathological specimens confirmed positive for a panel of biomarkers. These included the nuclear markers estrogen receptor (ER) and Ki-67, the cytoplasmic markers p16 and cytokeratin 5/6 (CK5/6), and the membranous markers human epidermal growth factor receptor 2 (HER2) and E-cadherin. Immunohistochemical staining was performed using the BOND-III platform to comprehensively assess the impact of a systematic gradient of antibody dilutions on staining quality. The resulting stains were evaluated by comparing the intensity, contrast, and degree of non-specific background across the different dilution levels. Staining scores were statistically compared among dilution groups. Differences in the rate of optimal staining were analyzed using Fisher's exact test.
Results: On the BOND-III platform, the optimal dilution ratio varied significantly for different antibodies. The ER, Ki-67, CK5/6, HER2, and E-cadherin antibodies achieved optimal staining at a 1:2 dilution, characterized by clear positive cell expression, clean background, absence of non-specific staining, and high contrast. Consistently, these five antibodies showed the highest immunohistochemical staining scores at a 1:2 dilution among all tested concentrations, with statistically significant differences compared to the neat group. In contrast, the p16 antibody demonstrated optimal staining at a 1:5 dilution ratio, showing well-defined positive cell localization and clear contrast, which was significantly superior to the neat antibody and all other dilution groups.
Conclusion: Our findings demonstrate that the optimal primary antibody dilution ratio for the BOND-III platform is antibody-dependent. Specifically, antibodies that are often used undiluted on other platforms may require further dilution on the BOND-III, with an optimal range typically falling between 1:2 and 1:5. This optimization not only improves staining quality by enhancing staining specificity and reducing background interference, but also reduces the reagent costs for routine clinical practice.

Keywords: immunohistochemistry; BOND-III; antibody; concentration

Introduction

Immunohistochemistry plays a vital role in modern pathological diagnosis and precision medicine [1]. The accuracy of clinical diagnoses and the scientific basis for treatment decisions are fundamentally reliant on the standardization and reliability of immunohistochemistry results. The recent proliferation of fully automated immunohistochemistry systems has substantially enhanced automation and process standardization across essential procedural steps, including tissue pretreatment, antigen retrieval, antibody incubation, and chromogenic detection [2,3]. This advancement has markedly diminished technical variability historically associated with manual methods, such as interoperator discrepancy and inconsistencies in timing and temperature control, thereby strengthening the reproducibility and reliability of staining outcomes [4].

However, within routine pathology laboratory practice, newly acquired lots or brands of primary antibodies necessitate rigorous performance validation and optimization before their application in clinical diagnostics. Establishing the optimal working concentration and staining conditions for a given antibody on a specific automated platform is imperative, as these parameters directly influence key quality indicators: staining specificity, signal intensity, and background clarity [5]. An excessive antibody concen-

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tration promotes non-specific binding and elevated background, potentially yielding false-positive interpretations. Conversely, an insufficient concentration risks signal attenuation or loss, leading to false-negative results [6]. Consequently, devising a scientific dilution protocol remains a central challenge in pathology quality assurance and standardization initiatives.

This study was performed using the Leica BOND-III fully automated immunohistochemistry platform. We investigated a panel of clinically pivotal biomarkers with distinct subcellular localizations: nuclear markers estrogen receptor (ER) and Ki-67, cytoplasmic markers p16 and cytokeratin 5/6 (CK5/6), and membranous markers human epidermal growth factor receptor 2 (HER2) and E-cadherin. We systematically diluted antibodies to compare their staining performance. The objective was to determine the optimal working concentration for each antibody within the BOND-III system and to formulate antibody-specific staining protocols. Our findings aim to improve the consistency, reliability, and diagnostic accuracy of immunohistochemistry assays, offering a foundation for enhancing laboratory standard operating procedures.

Methods

Specimen Collection

This study collected 64 archived paraffin-embedded tissue specimens from the Center of Precision Pathologic Diagnosis, Weifang People's Hospital between June and December 2023. Among these specimens, immunohistochemical staining was performed using antibodies against ER (n = 16), HER2 (n = 16), Ki-67 (n = 8), p16 (n = 8), CK5/6 (n = 8), and E-cadherin (n = 8). All selected specimens were pathologically confirmed. Serial sections (3 µm thick) were cut to ensure consistent experimental conditions. Specimen selection covered varying expression intensities to guarantee the representativeness and reliability of the results. Complete clinical data were available for all cases, and independent pathological assessment and review were conducted by two senior pathologists according to the diagnostic criteria in the fifth edition of the WHO Classification of Tumors [7]. This study was approved by the Ethics Committee of Weifang People's Hospital (Approval no. KYLL20251127-8) and every patient provided written informed consent. All procedures were performed in accordance with the Declaration of Helsinki.

Specimen Preparation

Tissue specimens were fixed in 10% buffered neutral formalin and processed routinely for paraffin embedding. Consecutive 3 µm sections (5–8 per block) were cut and assigned to the following groups: a (Neat), b (1:1), c (1:2), d (1:3). For p16, additional groups e (1:4), f (1:5), and g (1:6) were included.

Antibody Titration on the Leica BOND-III Immunohistochemistry Platform

In principle, ready-to-use antibodies do not require titration. However, if strong signals or non-specific staining are observed across various antigen retrieval methods, it may be necessary to dilute the antibody. In this study, preliminary experiments on the Leica BOND-III platform using neat antibodies resulted in intense staining with non-specific background. Therefore, after determining the optimal antigen retrieval method (EDTA buffer at pH 9.0, 100 °C, 20 min), the primary antibodies for ER (5278414001, Roche Diagnostics Co., Ltd., Shanghai, China), Ki-67 (OG-J009, Qiming Gene Technology Co., Ltd., China), CK5/6 (QG-J016, Qiming Gene Technology Co., Ltd., China), HER2 (IR322, LBP Co., Ltd., Guangzhou, China), and E-cadherin (QG-J127, Qiming Gene Technology Co., Ltd., China) were tested at the following dilutions: Neat, 1:1, 1:2, and 1:3. Since the p16 antibody (QG-J061, Qiming Gene Technology Co., Ltd., China) showed strong positive signals even at a 1:3 dilution, it was further tested at 1:4, 1:5, and 1:6 to determine the optimal dilution. The BOND immunostaining kit (DS9800, Leica Biosystems, Shanghai, China) used in this study includes the following components: peroxidase blocking reagent, post-primary reagent, polymer, DAB Part 1, DAB Part B, and hematoxylin.

Interpretation of Immunohistochemistry Results

Immunohistochemical staining results were evaluated independently by two board-certified pathologists, each with over five years of specialized experience in diagnostic immunohistochemistry. To eliminate assessment bias, both evaluators were blinded to the antibody dilution groups and all specimen identifiers throughout the scoring process. Staining was assessed based on the pre-defined scoring criteria detailed in Table 1, which encompassed staining intensity, specificity, and background clarity. For each antibody, the expected subcellular localization of positive staining was confirmed and recorded as follows: nuclear for ER and Ki-67, cytoplasmic for p16 and CK5/6, and membranous for HER2 and E-cadherin. According to the established criteria, a final composite score of 3 or 4 points was classified as optimal staining quality, deemed suitable for definitive diagnostic interpretation. Conversely, a score of 0 to 2 points was categorized as suboptimal staining, indicating insufficient reliability for clinical reporting.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Comparisons of immunohistochemical staining results among different antibody dilution groups were conducted using the Fisher's exact test. p value < 0.05 is considered statistically significant.

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Table 1. Immunohistochemistry scoring criteria.

Score	Assessment criteria
4	Precise localization, absence of non-specific staining, and clear contrast.
3	Precise localization with minor non-specific staining that does not affect result interpretation.
2	Precise localization, but with significant non-specific staining requiring explanatory notes.
1	Imprecise localization accompanied by significant non-specific staining.
0	Incorrect localization, severe non-specific staining, or false positive/negative results that lead to diagnostic misinterpretation.

Results

Immunohistochemical Staining Results at Different Antibody Dilutions

ER and Ki-67 are nuclear markers. At the undiluted concentration, nuclear positivity was pronounced but accompanied by significant non-specific cytoplasmic staining. At a 1:1 dilution, nuclear positivity remained distinct with only mild non-specific cytoplasmic staining and clear contrast. The 1:2 dilution yielded sharp nuclear staining without any non-specific staining. At a 1:3 dilution, nuclear positivity was reduced, albeit with no non-specific staining. At the same time, the immunohistochemical staining scores of ER and Ki-67 were highest at a 1:2 dilution ratio (Fig. 1a,b). CK5/6 is a cytoplasmic marker. The undiluted antibody produced intense cytoplasmic staining with prominent non-specific staining. At a 1:1 dilution, staining was acceptable with mild non-specific staining. A 1:2 dilution provided distinct cytoplasmic staining without non-specific staining and with clear contrast. At 1:3, staining intensity decreased in some areas without non-specific staining. The immunohistochemical staining scores of CK5/6 were highest at a 1:2 dilution ratio (Fig. 1c). HER2 and E-cadherin are membranous markers. Undiluted staining produced intense membranous staining with marked non-specific cytoplasmic staining. At a 1:1 dilution, membranous staining remained slightly overstained with mild non-specific cytoplasmic staining. The 1:2 dilution yielded moderate membranous staining with clear outlines and no non-specific staining. At a 1:3 dilution, membranous staining was weakened in some cells without non-specific staining. The immunohistochemical staining scores of HER2 and E-cadherin were highest at a 1:2 dilution ratio (Fig. 1d,e). p16 is a cytoplasmic marker. From neat to 1:4 dilutions, cytoplasmic staining was excessively intense with varying degrees of non-specific staining. At a 1:5 dilution, staining was moderate, clear, and free of non-specific staining. A further dilution to 1:6 resulted in decreased staining intensity in some cells, though without non-specific staining. The immunohistochemical staining of p16 achieved the highest immunohistochemical scores at a 1:5 dilution (Fig. 1f). In conclusion, the optimal dilution for ER, Ki-67, CK5/6, HER2, and E-cadherin antibodies was 1:2. For the p16 antibody, a dilution of 1:5 yielded the best results.

Table 2. Statistics on qualified staining rates at different antibody dilutions.

Antibody	Dilution ratio				Fisher's exact test
	Neat	1:1	1:2	1:3	p
ER					<0.001
Qualified staining	0	6	15	4	
Unqualified staining	16	10	1	12	
Ki-67					<0.001
Qualified staining	0	2	8	4	
Unqualified staining	8	6	0	4	
CK5/6					<0.05
Qualified staining	2	5	8	4	
Unqualified staining	6	3	0	4	
HER2					<0.001
Qualified staining	0	7	15	10	
Unqualified staining	16	9	1	6	
E-cadherin					<0.001
Qualified staining	0	3	8	5	
Unqualified staining	8	5	0	3	

Qualified Staining Rates at Different Antibody Dilutions

Statistical analysis of staining qualification rates across antibody dilutions revealed statistically significant differences for ER, Ki-67, p16, CK5/6, HER2, and E-cadherin ($p < 0.05$) (Table 2 and **Supplementary Table 1**). With the exception of p16, the five other antibodies achieved their highest staining qualification rates at a 1:2 dilution. In contrast, p16 demonstrated optimal performance at a higher dilution of 1:5. This disparity indicates that antibodies exhibit specificity in their optimal working concentration ranges on the BOND-III platform.

Discussion

Immunohistochemistry is essential for disease diagnosis, prognosis assessment, and treatment guidance [8–11]. With continuous technological advancement, fully automated immunohistochemistry systems have gained widespread adoption in pathology laboratories [12,13]. Their high level of automation, excellent reproducibility, and operational simplicity have substantially increased both the accuracy and efficiency of pathological diagnosis.

Immunohistochemistry is a multi-step experimental process whose staining quality is influenced by numerous

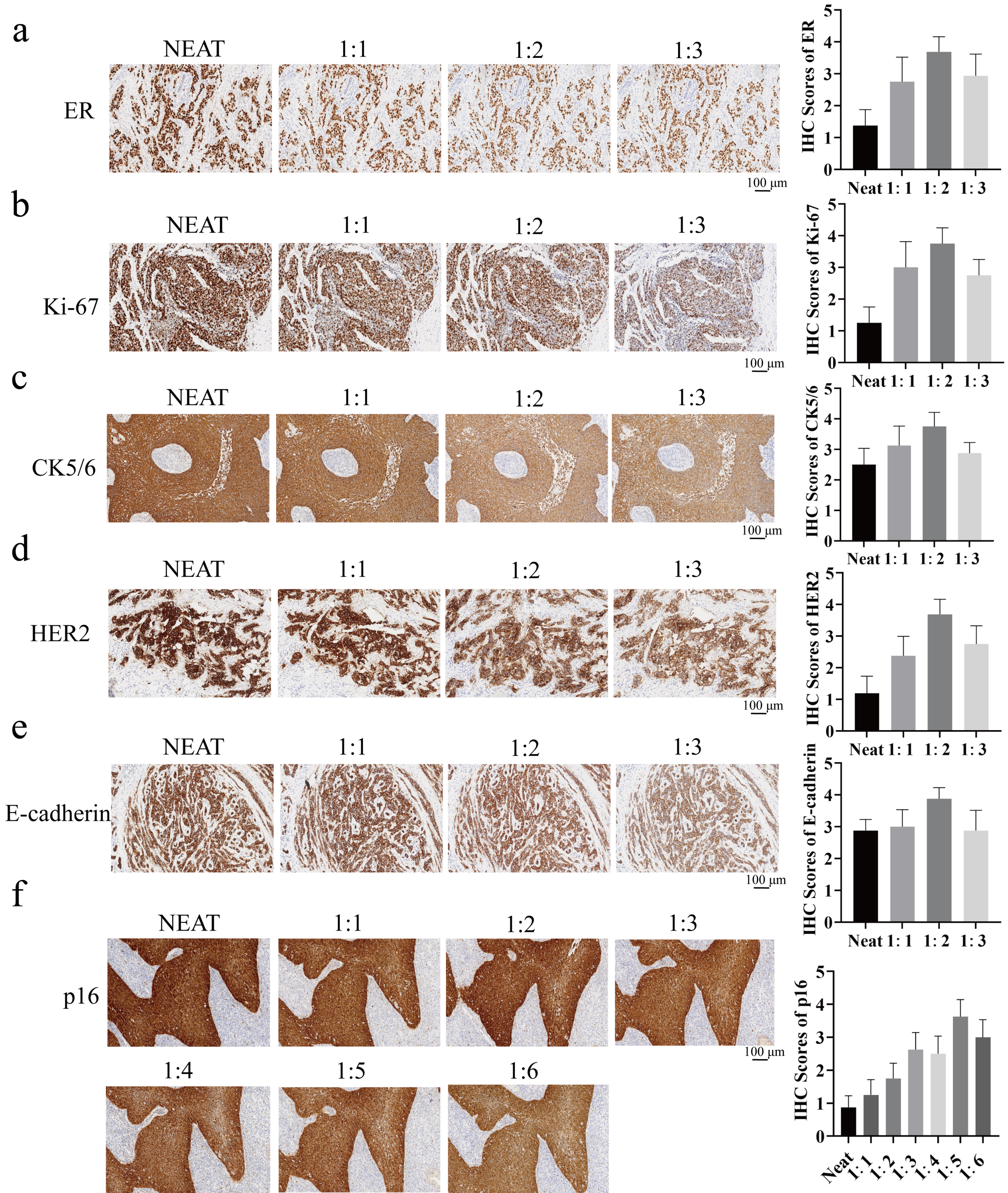

Fig. 1. Representative images and scores of immunohistochemical staining at different antibody dilutions. (a) Estrogen receptor (ER) (n = 16). (b) Ki-67 (n = 8). (c) Cytokeratin 5/6 (CK5/6) (n = 8). (d) Huamn epidermal growth factor receptor 2 (HER2) (n = 16). (e) E-cadherin (n = 8). (f) p16 (n = 8). Scale bar: 100 µm; 100× magnification.

critical factors [14–16]. From tissue specimen pretreatment, reagent selection, and quality control to instrument calibration and reaction condition optimization, each experimental step can significantly impact final staining outcomes [17,18]. Research indicates that the specificity and titer of primary antibodies, along with precise control of

DM DISCOVERY MEDICINE 2001

antigen retrieval duration, are the most crucial technical parameters for ensuring staining quality [19]. To guarantee the accuracy and reliability of test results, laboratories must conduct systematic performance validation when introducing new immunohistochemistry instruments, antibodies, or detection systems [20]. This process involves multi-factor optimization of various testing conditions via preliminary trials, followed by rigorous repeatability validation and result comparison to confirm the optimal experimental protocol [21]. A new system can only be deployed for routine clinical use after demonstrating that the staining results and performance metrics comply with clinical requirements. Studies have shown that laboratories must establish standardized validation protocols for newly introduced antibodies. Regardless of whether concentrated or ready-to-use antibodies are employed, working concentrations must be optimized through gradient dilution validation instead of relying on the manufacturer-recommended concentrations. This standardized procedure ensures optimal staining performance under specific laboratory conditions, providing a reliable foundation for subsequent clinical diagnosis.

During preliminary testing on the Leica BOND-III system, we observed that using primary antibodies at neat concentration produced overly intense staining with high non-specific background, which severely compromised interpretation accuracy. To address this, we first optimized key parameters, including antibody incubation time and antigen retrieval conditions. We then employed a gradient dilution method to identify the optimal working concentration for each antibody, aiming to establish a protocol that ensures accurate and reproducible immunohistochemical results. Our experiments determined the optimal dilution to be 1:2 for ER, Ki-67, CK5/6, HER2, and E-cadherin, and 1:5 for p16. Non-specific staining often arises from inadequate blocking or incomplete removal of endogenous enzymes like peroxidases and phosphatases. High background can also stem from improper antibody concentration or insufficient washing, while weak signals may result from suboptimal antigen retrieval or excessive antibody dilution [8]. In this study, after excluding variables related to blocking, retrieval, and washing, we found that high antibody concentrations produced strong positive signals but also introduced non-specific staining and elevated background. This is likely because antibody excess promotes non-specific binding. Conversely, low antibody concentrations yielded weak signals, probably due to insufficient antigen-antibody binding. The sensitivity of different primary antibodies to dilution depends on multiple factors, including antibody type, clone, and experimental conditions. In practice, systematic optimization tailored to specific requirements is essential to balance sensitivity and specificity. Our findings show that the optimal dilution for p16 antibody was 1:5, differing from the other five antibodies tested. This discrepancy may be related to the species origin of the antibodies: the p16 antibody is mouse-derived, whereas the other five are rabbit-derived. Lewis reported that diluted antibodies indeed reduce staining intensity but do not significantly compromise detection efficacy or performance, which can help conserve reagents and reduce costs [22]. Our results strongly support this view. An optimal concentration achieves the critical balance between sufficient signal intensity and minimal background, which is a prerequisite for accurate quantitative and semi-quantitative assessment in diagnostic pathology.

This study underscores the importance of optimizing primary antibody concentration on the Leica BOND-III platform, providing a critical reference for protocol design. It highlights that improper concentrations can lead to false results, thereby compromising the reliability of staining results. Furthermore, the work validates the economic advantage of optimization, demonstrating that it ensures staining quality while significantly reducing antibody consumption and cost. The established optimal dilutions (1:2 for most antibodies and 1:5 for p16) now constitute a validated component of the laboratory's standard operating procedure. Future studies should extend this systematic optimization to a broader panel of biomarkers. Furthermore, it is crucial to investigate whether these platform-specific optimal dilutions are consistent across diverse tissue types and fixation protocols. Such work would further solidify the robustness and generalizability of automated immunohistochemistry standardization.

Conclusion

In conclusion, our findings indicate that the optimal dilution ratios vary among different antibodies. Specifically, the optimal dilution was 1:2 for the ER, Ki-67, CK5/6, HER2, and E-cadherin antibodies, while it was 1:5 for the p16 antibody. Therefore, establishing a systematic protocol for antibody concentration optimization is essential for improving the quality of immunohistochemical staining. This approach can not only significantly enhance the accuracy of pathological diagnosis but also help reduce reagent costs while maintaining high staining quality.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

XTH and YTL: Methodology, Investigation, Formal analysis, Writing—original draft. XNF and HCK: Data curation, Visualization, Writing—review & editing. YXZ: Conceptualization, Supervision, Writing—review & editing, Funding acquisition. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experiments were approved by the Ethics Committee of Weifang People's Hospital (Approval no. KYLL20251127-8). Informed consent was obtained from all subjects. Human experimentation is consistent with the Declaration of Helsinki.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202638206.60.

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DISCOVERY MEDICINE 2001

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