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HIGH-THROUGHPUT SCREENING APPROACHES FOR IDENTIFICATION OF NEW BIOACTIVE COMPOUNDS

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Abstract

High-throughput screening (HTS) remains a central technology for transforming large chemical collections into experimentally validated starting points for drug discovery. Modern screening has evolved from single-concentration biochemical assays toward quantitative concentration-response formats, multiparametric phenotypic imaging, DNA-encoded libraries, virtual screening, machine-learning prioritization, and human-relevant cellular models. This structured review evaluates contemporary HTS approaches used to identify new bioactive compounds and examines the methodological factors that determine hit quality, reproducibility, mechanistic interpretability, and translational value. Peer-reviewed international literature published between 1999 and 2025 was synthesized with emphasis on assay validation, quantitative HTS, phenotypic screening, high-content analysis, computational triage, DNA-encoded libraries, artifact control, and active-learning approaches. The evidence indicates that no single platform is optimal for all discovery programs. Robust campaigns increasingly combine complementary technologies: biochemically precise primary assays, dose-response confirmation, orthogonal and counter screens, phenotypic profiling, cheminformatic filters, and early developability assessment. Z′-factor and control-based quality metrics remain fundamental, but modern screening quality also depends on concentration-response behavior, chemical tractability, assay-interference control, and reproducibility across orthogonal systems. Recent integration of omics and active learning can substantially enrich hit rates in complex phenotypic campaigns. The most effective strategy is therefore an integrated screening cascade that balances scale with biological relevance and uses explicit quality gates before compounds advance to hit-to-lead optimization.

Keywords: high-throughput screening; qHTS; phenotypic screening; high-content screening; bioactive compounds; DNA-encoded libraries; virtual screening; drug discovery

1. INTRODUCTION

The identification of chemically tractable molecules that modulate a therapeutically relevant biological process is one of the earliest and most consequential steps in small-molecule drug discovery. High-throughput screening was developed to test large compound collections in miniaturized, automated assays and thereby replace slow serial experimentation with parallel, data-rich discovery. Although the productivity of HTS has sometimes been criticized, industrial and academic experience shows that the technology is

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most useful when it is treated not as a stand-alone hit generator but as part of a broader scientific workflow integrating assay biology, medicinal chemistry, informatics, and validation (1,10).

Classical HTS usually measures the activity of each compound at one concentration against a defined molecular target. This architecture is scalable, experimentally straightforward, and highly compatible with robotic liquid handling. However, a single-concentration readout can confound true pharmacology with assay noise, compound aggregation, optical interference, or cytotoxicity. Quantitative high-throughput screening (qHTS) was introduced to address this limitation by testing compounds across concentration series, allowing activity to be interpreted from full concentration-response curves rather than a single endpoint (2). The approach increases information content and improves discrimination among high-quality, weak, and artifactual responses.

A second major evolution has been the renewed use of phenotypic screening. Instead of asking whether a molecule binds or inhibits a predefined target, phenotypic campaigns ask whether a compound corrects a disease-relevant cellular or organismal phenotype. Historical analyses suggest that phenotypic routes have contributed substantially to first-in-class medicines, particularly when disease biology is incompletely understood (4). Modern phenotypic drug discovery combines physiologically relevant models with automated microscopy, multiplexed biomarkers, omics, and target-identification technologies, thereby reducing the conceptual gap between target-based and system-level screening (5,6).

Screening technologies have also expanded beyond conventional microplates. High-content imaging captures multidimensional cellular phenotypes; DNA-encoded libraries provide access to extremely large chemical collections using affinity selection; virtual screening and machine learning prioritize compounds before experimental testing; and active-learning frameworks can iteratively select the most informative compounds for complex phenotypic assays (7,8,9,14). These platforms differ in throughput, cost, biological relevance, interpretability, and the type of chemical matter they can access.

The objective of this article is to synthesize the methodological principles and current evidence supporting modern high-throughput screening approaches for identification of new bioactive compounds, with particular attention to assay quality, hit confirmation, artifact control, technology integration, and emerging computational strategies.

2. MATERIALS AND METHODS

This article was prepared as a structured evidence-based review. Peer-reviewed international publications and authoritative methodological resources were selected for relevance to HTS design, qHTS, assay validation, phenotypic discovery, high-content screening, DNA-encoded libraries, computational screening, assay interference, and data-driven prioritization. The evidence base included seminal methodology papers and contemporary reviews or experimental studies published from 1999 through 2025.

Sources were prioritized when they described validated screening principles, compared screening strategies, introduced widely adopted quality metrics, or reported technological advances with clear implications for early drug discovery. Particular emphasis was placed on the Z-factor/Z′-factor framework for assay performance, concentration-response screening, orthogonal validation, interference filtering, image-based profiling, and modern

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strategies that combine experimental screening with machine learning or omics (2,3,7,11,14,15).

Because the purpose was methodological synthesis rather than meta-analysis of a single therapeutic outcome, results were organized by screening modality and by decision point in the discovery cascade. Evidence was compared across four dimensions: scalable chemical-space coverage, biological relevance, capacity to generate mechanistically interpretable hits, and vulnerability to false-positive or false-negative results. The resulting framework was used to formulate an integrated screening strategy suitable for early identification and prioritization of bioactive compounds.

3. RESULTS

3.1. Conventional HTS and Quantitative HTS

Conventional biochemical HTS remains particularly effective when the disease-relevant target is well validated and can be interrogated using a stable, miniaturized assay. Enzyme inhibition, receptor binding, protein-protein interaction, and reporter-based assays can be run in 384- or 1,536-well formats with automated dispensing and rapid optical detection. The strength of the approach is direct mechanistic interpretability: an active compound is linked to an explicitly defined biochemical event. Its main weakness is biological reductionism; a potent biochemical hit may fail in cells because of permeability, metabolism, target context, or compensatory signaling (1,10).

qHTS improves upon single-point HTS by testing compounds at multiple concentrations from the outset. Inglese and colleagues demonstrated that titration-based screening can efficiently classify biological activity across large libraries while reducing dependence on arbitrary single-concentration thresholds (2). Curve shape, maximal response, potency, and reproducibility collectively provide a richer basis for hit prioritization. qHTS is particularly useful when both weak agonism and strong inhibition may be biologically meaningful, or when the library contains compounds with heterogeneous physicochemical behavior.

Assay quality is central to both formats. The Z-factor described by Zhang, Chung, and Oldenburg integrates assay dynamic range with the variability of positive and negative controls (3). In practice, Z′ values of approximately 0.5 or greater are widely regarded as evidence of a robust assay, although screening decisions should also consider signal-to-background, coefficient of variation, control stability, plate effects, and inter-day reproducibility (3,13). A high Z′-factor does not guarantee pharmacological validity; it only demonstrates statistical separation of assay controls.

Table 1. Comparative characteristics of major screening modalities

Approach	Typical scale	Primary strength	Main limitation	Best use case

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Approach	Typical scale	Primary strength	Main limitation	Best use case
Biochemical HTS	10^4-10^6 compounds	High throughput; direct target linkage	Reduced biological context	Validated enzymes, receptors, defined molecular targets
qHTS	10^4-10^5 compounds with titrations	Immediate concentration-response information	Higher reagent/data burden	Libraries requiring potency and curve-quality classification
Phenotypic / HCS	10^3-10^5 compounds	Disease-relevant and multiparametric biology	Target deconvolution may be difficult	Complex disease biology; first-in-class discovery
DNA-encoded libraries	10^6-10^12 encoded members	Exceptional chemical-space coverage with low material use	Selection bias; requires target-compatible chemistry	Affinity ligand discovery for purified targets
Virtual / AI screening	10^6-10^9+ structures computationally	Low marginal experimental cost; prioritization	Model/domain bias; requires experimental validation	Pre-filtering, focused library design, hit enrichment
Complex cellular/organoid screens	10^2-10^4 compounds	High physiological relevance	Low throughput; higher variability/cost	Late hit confirmation and translational prioritization

3.2. Phenotypic Screening and High-Content Analysis

Phenotypic drug discovery can identify compounds that influence disease-relevant biology without requiring prior knowledge of the molecular target. This can be an advantage in disorders driven by network-level dysfunction, cellular state changes, or incompletely characterized mechanisms. Analyses of approved medicines and industrial discovery programs have therefore renewed interest in phenotypic approaches as a complement to target-centric discovery (4-6).

High-content screening extends phenotypic discovery by using automated microscopy to quantify multiple cellular features simultaneously. Rather than reducing the response to one scalar readout, image-based profiling can capture cell number, morphology, organelle

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structure, localization, texture, and other features at single-cell resolution. Caicedo and colleagues described a standardized analytical workflow that converts microscopy images into robust morphological profiles through segmentation, feature extraction, normalization, dimensionality reduction, and quality control (7).

The information density of high-content screening creates an opportunity for mechanism inference and compound clustering. Simm and colleagues showed that image-derived fingerprints from one high-throughput imaging assay could be repurposed to predict biological activity in other assays, demonstrating that cellular morphology can contain latent information about compound action beyond the original screen endpoint (15). This approach can reduce the number of compounds that must be tested in expensive secondary assays while preserving chemical diversity.

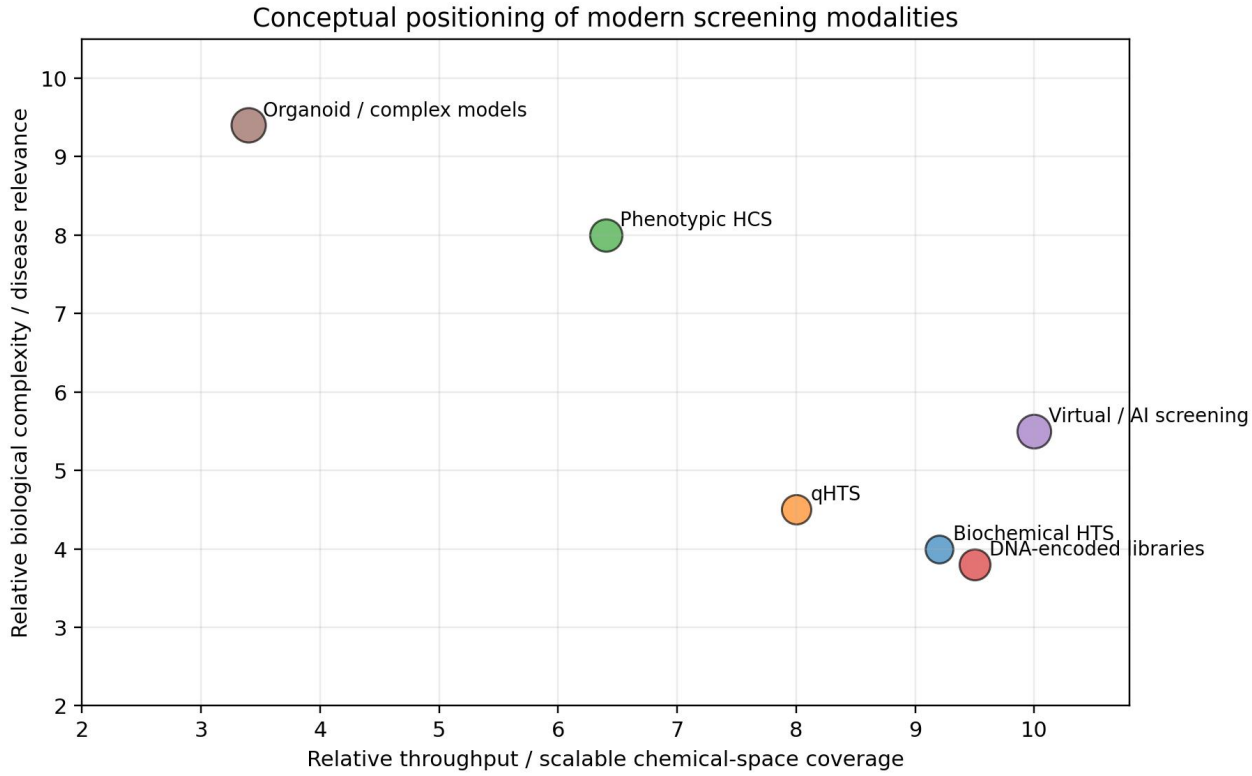


Figure 1. Conceptual positioning of modern screening platforms by throughput and biological complexity.

3.3. DNA-Encoded Libraries and Expanded Chemical Space

DNA-encoded library (DEL) technology addresses a different limitation of conventional HTS: the finite size of physically plated compound collections. In DELs, small molecules are covalently linked to DNA barcodes that record synthetic history, allowing very large libraries to be pooled and screened by affinity selection. The enriched DNA tags are sequenced to identify chemical structures preferentially retained by a target. Contemporary DEL platforms can access chemical space far beyond ordinary microplate libraries while using relatively small quantities of protein and compound (8).

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DEL screening is particularly powerful for discovery of binding ligands to purified proteins, including targets that are challenging for classical activity assays. However, affinity enrichment is not equivalent to functional pharmacology. Hits require off-DNA resynthesis and orthogonal biochemical or cellular confirmation, and selection conditions can favor nonspecific binders or chemotypes whose activity depends on the DNA-linked context. DELs are therefore best considered a complementary hit-generation technology embedded in a rigorous validation cascade rather than a replacement for functional HTS (8).

3.4. Virtual Screening, Machine Learning, and Active Learning

Virtual screening filters or ranks large chemical collections before experimental testing. Structure-based docking, ligand-based similarity, pharmacophore models, quantitative structure-activity relationships, and machine-learning models can all be used to enrich the proportion of experimentally active compounds. Even early reviews anticipated that virtual and experimental screening would become increasingly integrated because the two approaches solve complementary problems: computation can prioritize chemical space, whereas experiments establish biological truth (9).

Recent machine-learning strategies move beyond static prediction toward adaptive screening. In 2025, DeMeo and colleagues reported an active-learning framework that used transcriptomic signatures to prioritize compounds for complex phenotypic screening. Across two hematological discovery campaigns, the framework produced a reported 13- to 17-fold increase in phenotypic hit rate relative to baseline prioritization, followed by additional enrichment after laboratory-in-the-loop refinement (14). The significance of this work is not that computational models replace experiments, but that experimental budgets can be directed toward compounds expected to be maximally informative or biologically relevant.

AI-assisted screening nevertheless introduces new quality risks. Models can inherit biases from training datasets, overestimate confidence for compounds outside their applicability domain, and exploit assay-specific artifacts rather than causal biology. Prospective validation, chemically diverse test sets, calibration, and clear separation of training and evaluation data are therefore essential. High-performing models should ultimately be judged by their ability to improve prospective experimental hit rates and downstream chemical quality, not by retrospective metrics alone.

3.5. Assay Quality, False Positives, and Chemical Interference

High-throughput scale amplifies both good and bad assay behavior. Even a low false-positive rate can generate hundreds or thousands of misleading hits when very large libraries are tested. Artifacts arise from fluorescence interference, luciferase inhibition, redox cycling, detergent-sensitive aggregation, nonspecific reactivity, compound precipitation, or cytotoxicity. For this reason, a primary hit should be viewed as a hypothesis requiring confirmation rather than as a validated bioactive molecule.

PAINS filters were introduced to flag substructures associated with frequent activity across multiple assay systems, providing an early cheminformatic warning for potentially promiscuous compounds (11). These filters are useful but should not be treated as absolute exclusion rules because chemical context and assay mechanism matter. Experimental

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counter screens and orthogonal assays remain necessary. Similarly, unusually steep concentration-response curves may indicate stoichiometric or aggregation-related inhibition rather than conventional target binding, emphasizing the value of curve-shape analysis and reagent-concentration controls (12).

The best screening cascades therefore incorporate statistical, chemical, and biological quality gates. A compound that reproduces in an independent measurement principle, exhibits a plausible concentration-response relationship, survives interference and cytotoxicity counterscreens, and retains activity after fresh-powder confirmation has substantially higher credibility than a compound selected solely from a primary signal threshold.

Table 2. Quality-control parameters and common causes of screening error

Quality domain	Recommended check	Interpretation / purpose	Common failure detected
Assay separation	Z′-factor, control distributions	Evaluates dynamic range relative to variability	Weak separation, unstable assay window
Precision	CV, replicate agreement, plate controls	Measures technical reproducibility	Dispensing error, edge effects, day-to-day drift
Pharmacology	Concentration-response curve	Confirms potency, efficacy and curve shape	Single-point noise, steep/atypical responses
Detection artifact	Orthogonal readout	Confirms activity using different measurement principle	Fluorescence/luminescence interference
Nonspecific activity	Counter screen / selectivity panel	Tests activity unrelated to intended mechanism	Promiscuity, cytotoxicity, nonspecific binding
Chemical liability	PAINS/reactivity/aggregation review	Flags structures associated with recurrent assay interference	Reactive or colloidal false positives

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Quality domain	Recommended check	Interpretation / purpose	Common failure detected
Identity and purity	Fresh solid; analytical confirmation	Confirms that tested chemical is the intended entity	Degradation, contamination, incorrect structure

3.6. Hit Confirmation and Early Developability

The objective of a primary screen is not merely to maximize the number of active wells; it is to identify chemically credible starting points that can survive downstream optimization. Consequently, the transition from primary hit to validated hit should include resupply or resynthesis, concentration-response retesting, orthogonal activity confirmation, selectivity assessment, and evaluation of basic physicochemical properties. Solubility, permeability, metabolic stability, and obvious structural liabilities can be assessed early enough to prevent disproportionate investment in compounds that cannot become useful leads.

Hit quality is also influenced by chemical diversity. Selecting many closely related analogues from one dominant series may inflate apparent hit numbers while reducing the number of independent mechanistic hypotheses. Cluster-based selection, novelty analysis, and diversity-aware prioritization help maintain multiple chemical starting points. Conversely, a weak but structurally novel compound with reproducible mechanism-consistent activity may be more valuable than a highly potent but chemically problematic frequent hitter.

Table 3. Recommended hit-confirmation cascade after a primary screen

Stage	Key question	Typical action	Advance criterion
1. Re-test	Is the primary activity reproducible?	Repeat primary assay from independent aliquot	Consistent activity above predefined threshold
2. Dose response	Is activity concentration dependent?	Generate multi-point concentration-response curve	Plausible potency, efficacy and curve shape
3. Orthogonal assay	Is activity independent of detection technology?	Use alternative assay principle	Activity reproduced by independent readout
4. Counter screen	Is activity selective?	Test interference, cytotoxicity, related targets	No dominant nonspecific mechanism

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Stage	Key question	Typical action	Advance criterion
5. Chemical confirmation	Is the structure/purity correct?	LC-MS/NMR where appropriate; fresh material	Identity and purity acceptable
6. Early developability	Can the series be optimized?	Solubility, permeability, stability, liability review	Sufficient chemical and ADME tractability

3.7. Integrated Screening Strategy

The evidence supports a tiered rather than technology-exclusive strategy. A discovery program can begin with target-based HTS when target validation is strong, phenotypic screening when disease biology is complex, DEL selection when very broad ligand discovery is required, or virtual screening when structural or historical activity data permit computational enrichment. The primary platform should then be paired with complementary methods that address its characteristic weaknesses.

For example, target-based hits can be transitioned rapidly into cell-based functional assays to test biological context. Phenotypic hits can undergo target deconvolution and multi-omics profiling. DEL hits require functional off-DNA confirmation. AI-prioritized compounds require prospective wet-lab testing. Across all modalities, the common denominator is the same: multiple independent lines of evidence are needed before a screening signal is treated as a drug-discovery starting point.

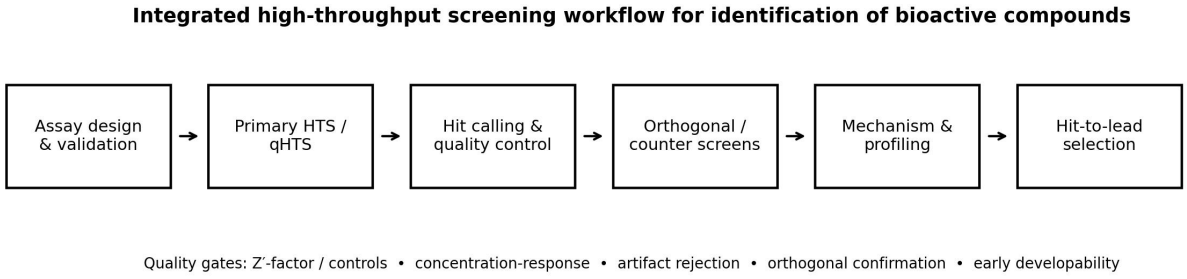


Figure 2. Integrated HTS cascade with sequential quality gates from assay validation to hit-to-lead selection.

Table 4. Strategic selection of screening technology according to discovery objective

Discovery situation	Preferred starting modality	Complementary technology	Rationale
Highly validated molecular target	Biochemical HTS or qHTS	Cellular confirmation; structure-based modeling	Maximizes mechanistic clarity and throughput

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Discovery situation	Preferred starting modality	Complementary technology	Rationale
Complex disease phenotype with uncertain target	Phenotypic HCS	Omics, target deconvolution, active learning	Captures network biology before mechanism is fully known
Need for very broad ligand search	DNA-encoded library	Off-DNA biochemical and cellular assays	Expands accessible chemical space dramatically
Large virtual library with structural data	Docking / ML virtual screen	Focused experimental HTS or qHTS	Reduces experimental burden and enriches likely actives
Expensive human-relevant model	Active-learning or image-based prioritization	Organoid/primary-cell confirmation	Uses computation to ration scarce experimental capacity

3.8. Human-Relevant Models, Reproducibility, and Future Screening Architecture

A major challenge in early drug discovery is the gap between highly scalable assays and the biological complexity of human disease. Primary cells, induced pluripotent stem-cell-derived systems, three-dimensional cultures, and organoids can provide more relevant tissue context than immortalized cell lines, but they are more expensive, heterogeneous, and difficult to automate at very large scale. A practical solution is a funnel architecture in which high-throughput methods are used to explore chemical space broadly and more complex human-relevant systems are reserved for confirmation and prioritization. This design treats biological complexity as a progressively increasing quality gate rather than as a requirement for every compound in the primary screen (5,6).

Reproducibility should be planned from the beginning of the screening campaign rather than assessed only after hits have been found. Plate maps should include appropriate controls distributed across the experimental run; assay performance should be monitored for drift; replicate strategy should be matched to assay variability; and raw as well as processed data should be retained for re-analysis. In high-content screening, reproducibility also depends on image segmentation, feature normalization, batch correction, and the stability of cellular state across plates and days (7). These considerations become especially important when machine-learning models are trained on screening data because systematic experimental bias can be learned as if it were a biological signal.

Future screening programs are likely to combine three layers of information: chemical structure, experimentally observed cellular state, and adaptive computational prediction. Structure-based models can identify plausible molecules before testing; imaging or omics can provide a high-dimensional description of what compounds actually do to cells; and active-learning algorithms can then use those observations to choose the next most

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informative compounds. The 2025 transcriptomics-based active-learning study provides a contemporary example of this closed-loop concept, while earlier image-based studies demonstrated that phenotypic profiles can predict activity across assays (14,15). The resulting architecture is neither fully virtual nor purely experimental. Instead, it is an iterative human-supervised system in which models reduce unnecessary experiments and experiments continually recalibrate the models.

For pharmacological screening, the implication is that throughput should no longer be treated as the dominant measure of technological sophistication. A campaign that screens fewer compounds but generates concentration-response data, orthogonal confirmation, high-dimensional phenotypes, and early developability information may produce a more valuable set of starting points than a much larger single-point campaign. The optimal endpoint is therefore not the greatest number of primary hits, but the greatest number of reproducible and chemically tractable hypotheses that can be advanced efficiently into mechanism studies and medicinal-chemistry optimization.

4. DISCUSSION

Modern HTS is best understood as an information-generation system rather than a robotic process for maximizing assay counts. The most important change over the past two decades is the shift from throughput alone toward information quality. Early HTS programs often emphasized the number of compounds screened, whereas contemporary programs increasingly emphasize concentration-response behavior, multidimensional phenotypes, chemical diversity, orthogonal confirmation, and prospective prediction (1,2,5,7).

Target-based and phenotypic screening should not be viewed as mutually exclusive philosophies. Target-based assays provide direct mechanistic linkage and are often ideal for well-established molecular hypotheses. Phenotypic assays provide integrated biological context and can discover unexpected mechanisms. A rational program can use both sequentially: target-based screening for efficient chemical expansion followed by disease-relevant functional confirmation, or phenotypic screening followed by mechanistic deconvolution. The choice should be driven by the maturity of disease biology and the nature of the therapeutic hypothesis rather than by platform availability alone (4-6).

The rapid growth of DNA-encoded libraries and computational screening extends accessible chemical space but also changes the definition of 'screening.' In a DEL selection, a physical library of millions or billions of tagged molecules may be interrogated in a pooled format. In virtual screening, the library may exist only in silico until a small subset is ordered or synthesized. These methods can dramatically increase search efficiency but shift the bottleneck toward validation. In both cases, the rate-limiting question becomes whether enriched or predicted molecules demonstrate reproducible functional activity under independent experimental conditions (8,9).

High-content imaging and machine learning create a similar shift from measurement scarcity to data abundance. A single imaging experiment can generate hundreds or thousands of features per cell, enabling mechanism inference and compound clustering but also introducing substantial analytical complexity. Reproducible preprocessing, batch correction, appropriate controls, and independent validation are therefore as important as

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microscope performance. Image-based profiling is especially promising because the resulting representations can be reused across discovery programs rather than being limited to one target or endpoint (7,15).

The 2025 active-learning study by DeMeo and colleagues illustrates a likely direction for future screening: iterative integration of experimental measurements with models that decide what to test next (14). Such systems can reduce the number of compounds required to explore large biological and chemical spaces. However, the value of active learning depends on careful prospective design. If the phenotype, training data, or model objectives are poorly aligned with therapeutic biology, computational efficiency can simply accelerate the wrong experiment. Human scientific judgment therefore remains necessary to define phenotypes, choose quality controls, interpret unusual compounds, and decide when model confidence is biologically plausible.

Assay artifacts remain a persistent threat regardless of computational sophistication. PAINS, aggregation, fluorescence interference, redox effects, and cytotoxicity can generate apparently convincing signals in automated screens (11,12). Importantly, cheminformatic alerts are not substitutes for experiments. A structural flag should trigger further investigation, not automatically classify a molecule as inactive. Orthogonal assays, detergent sensitivity where relevant, target counterscreens, and fresh-compound confirmation provide stronger evidence than structure-based filtering alone.

A limitation of this review is that screening productivity varies strongly across target classes and therapeutic areas, making direct numerical comparison of platforms difficult. In addition, many industrial datasets are proprietary, so published examples may overrepresent successful campaigns. Nevertheless, the methodological literature consistently supports the same principle: screening success is determined by the design of the complete cascade, not by the nominal capacity of the primary platform.

5. CONCLUSION

High-throughput screening remains indispensable for the identification of new bioactive compounds, but its modern form is substantially broader than classical single-point microplate screening. qHTS provides concentration-response information at scale; phenotypic and high-content approaches capture complex biology; DNA-encoded libraries expand accessible chemical space; virtual and AI-assisted screening enrich experimental testing; and active learning enables adaptive allocation of screening resources.

The central requirement across these technologies is rigorous validation. Reliable discovery requires robust assay statistics, concentration-response confirmation, orthogonal readouts, interference and selectivity counter screens, chemical identity verification, and early evaluation of developability. The strongest contemporary strategy is therefore an integrated screening cascade in which complementary technologies are selected according to the biological question and connected by explicit quality gates. Such a framework improves the probability that a primary screening signal will mature into a reproducible, mechanistically meaningful, and chemically tractable lead series.

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