Published in final edited form as:

Methods Mol Biol. 2016 ; 1413: 349–363. doi:10.1007/978-1-4939-3542-0_22.

Engineering and functional analysis of mitotic kinases through chemical genetics

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Abstract

During mitosis, multiple protein kinases transform the cytoskeleton and chromosomes into new and highly dynamic structures that mediate the faithful transmission of genetic information and cell division. However, the large number and strong conservation of mammalian kinases in general (1) pose significant obstacles to interrogating them with small molecules, due to the difficulty in identifying and validating those which are truly selective. To overcome this problem, a steric complementation strategy has been developed, in which a bulky “gatekeeper” residue within the active site of the kinase of interest is replaced with a smaller amino acid, such as glycine or alanine. The enlarged catalytic pocket can then be targeted in an allele-specific manner with bulky purine analogs (2, 3). This strategy provides a general framework for dissecting kinase function with high selectivity, rapid kinetics, and reversibility. In this chapter we discuss the principles and techniques needed to implement this chemical genetic approach in mammalian cells.

Keywords

Bump-hole; chemical genetics; analog-sensitive; kinase engineering; genome editing

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¹Fragments can also be ordered as synthetic gene blocks, obviating the need to locate a full-length and mutation-free cDNA clone as a PCR template.

⁵Choosing an appropriate selection marker is relatively straightforward as it depends on the sensitivity of your chosen cell line. For human telomerase reverse transcriptase (hTERT)-immortalized retinal pigment epithelial (RPE) cells we recommend using G418 (500 µg/ml) selection. Puromycin (10–15 µg/ml) and blasticidin (5–10 µg/ml) can be also be used but are less effective. HCT116 cells sensitive to G418 (500 µg/ml), blasticidin (5–10 µg/ml) and puromycin (2 µg/ml).

⁶Optimizing transgene expression can be achieved through promoter selection. Promoters such as CMV and SV40 are capable of high levels of expression and should be chosen for highly abundant kinases. Low abundance kinases can be expressed using the EF1α promoter. We also recommend screening clonal populations by western blotting for appropriate transgene expression.

⁷Although affinity and large localization tags such as GFP and mCherry can in some case cause problems with protein function, we recommend incorporating tags into your transgene. We generally include both an affinity and localization tag, as they can be extremely useful when studying the different properties of the active and inactive kinase.

⁸In many cell lines (including HCT116), a large fraction of retroviral integrations that are initially transcriptional active become silenced over time, unless selective pressure is applied. To counteract the silencing of retroviral transgenes we recommend using plasmid where the transgenes are linked to the selection marker (e.g., an ires-neo or ires-puro plasmid such as pQCXIN and pQCXIP).

1. Introduction

1.1 General Principles of Chemical Genetics

Analog-sensitive (as) kinase engineering was developed by the Shokat laboratory based on an insightful observation made while studying the interaction between the Src family kinases and the ATP-competitive inhibitor, PP1 (3–5). The structure of PP1 bound to the Src family kinase, Hck, revealed the inhibitor mimics the adenine ring of ATP in its interactions within the catalytic pocket. The orientation of the inhibitor within the pocket provided the foundation for building PP1 analogs with enlarged substituents capable of specifically targeting kinases containing small glycine or alanine gatekeeper residues (4, 6). The steric complementation provided by the gatekeeper mutation can be visualized in structure of the tyrosine kinase Hck bound to PP1 or the bulky PP1 analogs 1-NAPPI, 1-NM-PP1 or 3-MB-PP1 (Fig 1B,D). Although PP1 can fit within the wild-type Hck kinase, the bulky substituents in the PP1 analogs are oriented toward the gatekeeper and only fit within the pocket created by the gatekeeper mutation (6).

The high conservation of the bulky gatekeeper residue is key to ensuring the specificity and versatility of this approach. The chemical genetic strategy provides significant kinetic and specificity advantages over alternate methods used to study the function of protein kinases (see Table 1). Since native kinases are resistant to bulky purine analogs, cells expressing a wild-type kinase of interest (KOI) can be used to control for any off-target effects of the bulky purine analogs, allowing the phenotypic effects of the inhibitors to be linked to the genotype of the kinase, providing a significant advantage over traditional pharmacological inhibitors (7, 8).

The gatekeeper residue is a key determinant of kinase inhibitor sensitivity, and substituting the gatekeeper residue can provide resistance to pharmacological inhibitors directed towards the wild-type kinases. For example, Plk1^{as} cells are not only sensitive to certain bulky purine analogs but also cross-resistant to two other Plk1 inhibitors (BI-536 and TAL) currently being developed as cancer chemotherapeutics (9). Similarly, the BCR-ABL gatekeeper residue is a hotspot for resistance mutations in patients with chronic myelogenous leukemia who relapse during imatinib (Gleevec) therapy (10). In this case, acquisition of a larger gatekeeper results in the steric blockade of imatinib from the active site. Inhibitor resistance also provides useful differentiation of the on-target and off-target effects of small molecules, and especially important when assessing kinase inhibitors as therapeutic agents (Fig 1A) (11, 12).

The chemical genetic strategy has proven effective for protein kinases in diverse model organisms (2, 6). In mammals a variety of cell types can be used, including bone marrow derived hematopoietic cells, neurons and tumor cell lines (11, 13–15), allowing for flexibility and alignment with the underlying biological question or downstream applications. This chapter outlines the key steps for generating a chemical genetic system in human somatic cells (Fig 2).

2. Materials

2.1 Cell Culture and Production of Retroviral Particles

1. Phoenix cells (ATCC)
2. Medium for Phoenix cells:
D-MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Omega Scientific) and 0.1 mg/ml penicillin streptomycin (Life Technologies)
3. Hank's balanced salt solution (HBSS; Life Technologies)
4. OptiMEM medium (Life Technologies).
5. 0.05% Trypsin-EDTA (Life Technologies)
6. Tissue culture treated T-25 and T-75 flasks (Corning)
7. Tissue culture-treated 96-, 12- and 6-well plates (Corning)
8. G418 (Gemini Bio-Products)
9. Puromycin (Sigma-Aldrich)
10. Blasticidin (Sigma-Aldrich)
11. 0.45 micron syringe filters (EMD Millipore)
12. 10 ml syringes (Fisher Scientific)
13. Eugene 6 transfection reagent (Promega)
14. Polybrene (Sigma-Aldrich)
15. VSV-G plasmid (Addgene #8454)
16. Crystal violet staining solution (Crystal Violet 0.05% w/v) formaldehyde (1%) methanol (1%) in PBS.

2.1 Molecular Biology Reagents

- 1 Phusion polymerase kit (NEB)
- 2 Gibson assembly mix (NEB)
- 3 Primers (Integrated DNA Technologies)
- 4 10 mM dNTPs (Affymetrix)
- 5 Mineral oil (Sigma-Aldrich)
- 6 Ultrapure water (RNase/DNase-free) (Sigma-Aldrich)
- 7 Tris-EDTA (TE) buffer.
- 8 PCR purification kit (Macherey Nagel)
- 9 Plasmid DNA purification kit (Macherey Nagel)

- 10 Carbenicillin and Kanamycin (Sigma-Aldrich)
- 11 Luria Broth (LB) media (Fisher Scientific)
- 11 Standard DNA gel electrophoresis reagents and apparatus.

2.3 Chemical Genetic, Cell Staining and Protein Reagents

- 1 1NM-PP1 and 3MB-PP1 (EMD Millipore)
- 2 Staralog inhibitors (2, 16)
- 4 Phosphospecific substrate antibody (if available)
- 5 MPM-2 antibody (EMD Millipore)
- 6 Alexa fluor 488 labeled goat anti-mouse IgG (H+L) antibody (Life technologies)
- 7 Propidium Iodide
- 8 Bovine serum albumin (BSA) (Sigma-Aldrich)
- 9 Standard SDS PAGE reagents and apparatus

3. Method

3.1 Kinase Design and Engineering

The first step in engineering an analog sensitive kinase allele is to identify the gatekeeper residue. This side chain has been annotated for over 7000 protein kinases in different model organisms in the Kinase Sequence Database (<http://sequoia.ucsf.edu/ksd/>). It can also be identified by aligning the sequence of your KOI with c-Src using Clustal Omega (<http://www.clustal.org/omega/>) (17). The gatekeeper corresponds to T338 in the c-Src kinase and is commonly a leucine, methionine, phenylalanine or threonine in other kinases. The residues surrounding the gatekeeper may also help identify the correct position. Two hydrophobic amino acids are located at the -2 and -1 (I/L/M/F and V/F/L/I) positions and an acidic residue (E/D) can be found at the +1 position (Fig 1C). Atypical kinases may require additional structural modeling to accurately locate the gatekeeper. Detailed guidelines for identifying the gatekeeper within atypical kinases have been provided elsewhere (2).

The next step is to replace the gatekeeper with a smaller amino acid. In most cases, glycine is the preferred substitute, as this offers maximal enlargement of the ATP-binding pocket and opportunity for allele-specific inhibition. However, for 15 to 30% of kinases this alteration compromises activity and function unduly, in which case alanine can be used (2). For example, an alanine substitution (M602A) was better tolerated than glycine for human Mps1 (18). Some kinases may require a second-site suppressor to restore or improve the function of a gatekeeper-mutated kinase (2, 19). For example, human Plk1 requires an additional mutation (C67V) to tolerate glycine in place of its normal gatekeeper (L130G) (20). In cases where both glycine and alanine are poorly tolerated, another option is to engineer an electrophile-sensitive (ES) kinase, which involves replacing the gatekeeper with cysteine, a larger amino acid that is often well tolerated (21). One limitation of the ES approach is that kinase inhibition is irreversible, precluding experiments that depend on

inhibitor washout to reactivate the kinase. Finally, some gatekeeper mutants with reduced activity have also been rescued chemically using the bulky purine analog N6-benzylaminopurine (6-BAP). Addition of 6-BAP to cells expressing the Cdk2 (F80G) analog sensitive kinase was shown to restore both cyclin A binding and preserve kinase activity in culture (22).

The gatekeeper mutation(s) can be introduced into the KOI using conventional site-directed mutagenesis (23). This approach can be more challenging to perform in large mammalian expression vectors, especially plasmids containing long terminal repeats (LTRs) for viral delivery. Here we describe an approach to assemble the desired mutations into a final expression vector by combining conventional overlapping PCR with Gibson assembly. PCR fragments containing the desired gatekeeper mutations are assembled into the final linearized vector using overlapping homology. The example below describes how to generate the gatekeeper mutation and introduce the mutated KOI into a suitable expression vector in one step.

3.2 Gatekeeper Mutagenesis and Cloning via Gibson Assembly

Gibson assembly allows 5 or more fragments to be joined in one step. The approach is flexible and seamless, avoiding the need to introduce new restriction enzyme sites or site-specific recombination sequences. Briefly, the KOI is first amplified in two overlapping N and C terminal fragments using primers that contain ~20 bp of overlap with their neighbors, as well as the desired gatekeeper mutation (Fig 3A). DNA fragments can be combined as crude PCR products, obviating the need for gel purification. In the Gibson assembly reaction, three different enzymes (a 5' exonuclease, a DNA polymerase, and a DNA ligase) serve to cross-anneal, fill in, and ligate the fragments and vector, thus creating the final expression construct (Fig 3B). A sample protocol is provided below.

- 1 Using primers that meet the specifications above, amplify the two KOI fragments using a high fidelity PCR enzyme. The wild-type kinase can be amplified in a single fragment. The following conditions are suitable for amplification with Phusion polymerase.

	1X reaction (μl)
5X Phusion buffer	4
10 mM dNTPs	0.4
PCR water	12.4
Phusion polymerase	0.2
10 μM F primer	1.0
10 μM R primer	1.0
Template cDNA (10 ng)	1.0
total	20 μl

Overlay with mineral oil and cycle:

1 × 98°C 30 sec

$30 \times (98^{\circ}\text{C } 10 \text{ sec}/55^{\circ}\text{C } 30 \text{ sec}/72^{\circ}\text{C } 15\text{--}30 \text{ sec/kb})$ (See Note 2)

$1 \times (72^{\circ}\text{C } 20 \text{ sec})$

4°C hold

- 2 Run a small amount ($\sim 2 \mu\text{l}$) on a 1.0% TAE-agarose gel. Estimate the DNA concentration from band intensity (see Note 3). If one major product is obtained, use the crude PCR product directly for cloning; otherwise, purify by gel extraction and quantify by nanodrop or gel electrophoresis.
- 5 Linearize the expression vector at the desired insertion site, gel purify, and quantify as above.
- 4 Set up Gibson assembly reactions, together with a negative (no-insert) control (see Note 4):

	1X reaction (μl)
Linearized vector (0.02 pmol)	1.0
N-terminal kinase fragment (0.06 pmol)	1.0
C-terminal kinase fragment (0.06 pmol)	1.0
2 $\times$ Gibson assembly mix	3.0
total	6 μl

- 5 Overlay with mineral oil and incubate in thermocycler at 50°C for 15–60 min.
- 6 Transform 1–2 μl of the Gibson assembly reaction into DH10B or an alternate *E. coli* strain.

3.2 Replacing the Endogenous Kinase with the Analog-Sensitive Allele

As analog-sensitive kinases are generally recessive, their usage requires the endogenous kinase pool to be completely and permanently removed from the cell. This objective can be accomplished using AAV-mediated gene targeting (18, 20, 24), stable RNAi (25) or RNA-guided nucleases (26, 27). One option is to ‘knock in’ the gatekeeper substitution at the KOI gene locus, thus maintaining endogenous expression control (15, 22). A limitation of this approach is that for many kinases it is difficult to predict *a priori* which gatekeeper substitution will work best (i.e., maintain activity and support cellular function in the absence of analogs, yet be fully and effectively inhibited in their presence). Furthermore, this approach becomes infeasible if second-site suppressor mutations in a distant exon are also necessary. While *in vitro* kinase assays can provide some guidance on these points, they

²For primers having an annealing temperature 72°C (as determined for their template-specific 3' ends), a 2-step thermocycling protocol is recommended.

³Nanodrop readings are unreliable for crude PCR products because of contaminating primers and dNTPs.

⁴Calculations for Gibson assembly:

$$\text{Vector } 0.02 \text{ pmol} = 13.2 \text{ ng} \times \text{size kb}$$

$$\text{N-terminal insert } 0.06 \text{ pmol} = 39.6 \text{ ng} \times \text{size kb}$$

$$\text{C-terminal insert } 0.06 \text{ pmol} = 39.6 \text{ ng} \times \text{size kb}$$

may not replicate the physiologic situation accurately, due to the use of artificial substrates and reaction conditions, as well as omission of kinase scaffolds, feedback controls, and subcellular localization. Indeed, analog-sensitive versions of human Plk1 (20) and yeast Cdc28/Cdk1 (3) have reduced biochemical activity as measured *in vitro* but fully reconstitute kinase function (including the quantitative phosphorylation of substrates) *in vivo*. For these reasons, we prefer to introduce analog-sensitive kinases as transgenes, allowing for rapid *in vivo* testing of different gatekeeper (and if needed, second-site suppressor) mutations. Depending on the nature of the expression system, constructs can be delivered through stable transfection or virus-mediated transduction. Below we provide a protocol for generating pantropic retroviral particles that can transduce in all cell types.

- 1 For small-scale retroviral production, transfect a T-25 flask of Phoenix cells. These HEK293 derivatives express the gag and pol genes from Moloney murine leukemia virus (MoMuLV) necessary for virion assembly and replication. Grow cells (in DMEM + 10% FBS + pen/strep) to approximately 50–60% confluence.
- 2 Dilute 3 µg of plasmid DNA (2 µg of retroviral construct + 1 µg of the pVSV-G pantropic envelope vector) into 400 µl of OptiMEM.
- 3 Add 9 µl of FuGene 6 to diluted DNA and mix gently.
- 4 Incubate DNA/FuGene 6 mixture at room temperature for 20 minutes.
- 5 Replace medium on cells with 4–5 ml fresh media.
- 6 Add transfection mix onto cells dropwise. Gently tilt flask to distribute complexes onto the entire cell monolayer.
- 7 Incubate cells overnight in tissue culture incubator. The Phoenix cells must be handled with BL2 safety precautions.
- 8 Discard old medium 24 hours after transfection and replace with fresh media.
- 9 Seed target cells into multiple wells in a 12 well plate at a confluency of ~10–15%. The following day, when the cells reach ~20–30% confluence, they will be ready for retroviral infection.
- 9 Collect retrovirus-containing supernatant 48 hours after transfection into a 15 ml conical tube. Spin out cell debris at 1000 × g for 5 minutes and/or filter through 0.45 micron syringe filter.
- 10 Replace media on target cells with 1 ml of fresh antibiotic free media and add 1ml of filtered retrovirus-containing supernatant.
- 11 Add polybrene to final concentration of 4 µg/ml during transduction (see Note 9).
- 12 Incubate for 8 hours to overnight.

⁹Polybrene is a positively charged polymer that reduces charge repulsion between the virus and the cellular membrane. Polybrene to a final concentration of 2–10 µg/ml is suitable for most human cell lines; however, some cell types are sensitive to polybrene. The optimum concentration may need to be determined empirically. Exposure to Polybrene (>24 hr) will increase its toxicity to cells.

- 13 Replace media with fresh complete medium and allow cells to recover after infection and expand into T-25 flask when confluent (~1–3 days).
- 14 Split cells into a T-75 flask and begin selecting for transduced cells once cells have attached (at least 24 hours later). Adding selection sooner may delay their recovery from the viral infection.
- 15 When using expression constructs encoding GFP, you should be able to detect expression by fluorescence microscopy 1–2 days after infection.
- 16 Evaluating transgene expression by Western blotting, immunofluorescence microscopy in the bulk population. It is also a good idea to sort GFP-positive cells by flow cytometry or isolate a clonal population with appropriate transgene expression.

3.3 Screening Analog-Allele Pairs for Optimal Specificity and Penetrance

Once the endogenous kinase has been replaced with an analog-sensitive version, the next step is to identify the optimal analog (that is, the compound that best discriminates the analog-sensitive and wildtype kinase alleles). A number of suitable analogs based on the PP1 scaffold (e.g., 1-NA-PP1, 1-NM-PP1 and 3-MB-PP1) have been used successfully. In addition, a new series of Staralog inhibitors (based on staurosporine as a general kinase-inhibiting scaffold) have been developed (16). Thus there are multiple options for researchers seeking to identify the most penetrant inhibitor among these small molecules. It is possible to *in vitro* screen a panel of inhibitors using purified wild-type and analog-sensitive kinases with peptide or protein substrates and gamma-32P-ATP. However, the gold standard for screening a panel of inhibitors is to measure kinase inhibition in cells by immunoblotting with a phospho-specific substrate antibody and/or assessing a biologically relevant phenotype. Ideally, the bulky purine analogs will possess greater than 10 fold selectivity for the AS kinase. However, in many instances a phospho-specific substrate antibody is not available. In such cases, phenotypic screening provides a facile and physiologically relevant means of identifying the most effective allele-inhibitor pair.

3.4 Assessing Susceptibility to Growth Inhibition Using Crystal Violet Staining

To assess whether the analog-sensitive cell line is susceptible to growth inhibition by bulky purine analogs, we suggest that researchers begin with a simple growth assay measured by crystal violet stain to assess whether bulky purine analogs can fully abrogate the KOIs function *in vivo*. This simple assay is extremely easy and informative and can be applied to screen multiple allele and drug combinations for dose-dependent growth inhibition. We have used this assay previously to demonstrate the specificity of 3-MB-PP1 and 1-NM-PP1 towards Plk1^{AS} and Mps1^{AS} cells (18, 20) (Fig 4).

- 1 Plate ~2000 cells/well in a 24 well plate. A good starting point is to test serial 4 fold dilutions of the bulky purine analogs 1NM-PP1 and 3-MB-PP1 across each row (0, 0.078 μ M, 0.3125 μ M, 1.25 μ M, 5.0 μ M, 20.0 μ M). At the highest concentration (~20 μ M), many cell lines such as HCT116 and RPEs will display mild growth inhibition (See Note 10).

- 2 After 6–10 days remove growth media (do not wash cells) and stain cells with 1 ml of crystal violet staining solution for 20 minutes.
- 4 Remove staining solution and save
- 5 Wash each plate individually by dipping into 5 L beaker of tap water.
- 6 Air dry each plate upside down over paper towel.

3.5 Rapid Assessment of M Phase Arrest or SAC Override By Flow Cytometry

Mitotic kinases are generally required to either satisfy or activate the spindle assembly checkpoint (SAC), which blocks anaphase and mitotic exit if kinetochore-microtubule attachments are absent or incorrect. Consequently, when starting to work with a new chemical-genetic system is useful to know whether kinase inhibition results in prolonged mitotic arrest, or alternatively overrides mitotic arrest induced by microtubule poisons or kinesin-specific inhibitors. This can be determined by flow cytometry on fixed cells stained with a mitosis-specific marker (see below) as well as time-lapse microscopy. The former approach is technically easier and scales readily to a large number of samples, while the latter approach provides superior temporal resolution and is less sensitive to perturbations in the rate of mitotic entry.

1. Grow cells in a T-75 flask to approximately 60 – 70% confluency. Treat cells with the bulky purine analog of choice in the presence or absence of nocodazole (16 μ M) for 8 – 12 hrs.

In addition to your samples, keep an extra flask to serve as a negative control (2nd antibody only staining).
2. Save mitotic cells by decanting the tissue culture medium into 50 ml tubes. Wash cells in 10 mls of PBS and collect into the same 50 ml tubes.
3. Remove adherent cells with 2 mls of trypsin and collect the cells by centrifugation at 900 g for 5 mins. Combine cells from step 2 and step 3.
4. Resuspend the cell pellets with 900 μ L PBS. Add 2.0 mls cold (-20° C) ethanol to cell suspension in a drop-wise manner while gently vortexing. Fix cells overnight at -20° C. Fixed cells can be stored at -20° C for up to a two months.
5. Pellet cells at 900g for 5 mins. Remove the ethanol supernatant carefully and resuspend the pellet in 500 μ L in PBS + 0.2 % Triton X-100. Transfer cells into a 1.5 ml microfuge tube and collect them by centrifugation at 900g for 5 min.
6. Resuspend the cell pellet with 500 μ L blocking buffer (PBS +0.2 % Triton X-100 + 3% BSA) for 10 mins. Centrifuge at 900g for 5 mins and remove the supernatant.
7. Resuspend cells with 500 μ L blocking buffer + 1.0 μ g anti-phospho-MPM-2 antibody. Incubate for 1hr at room temperature. Mix gently every ~10 mins.

¹⁰The concentration range is a starting point. We advise that follow up experiments be performed that sample concentrations closer to the effective dose identified in this range. The assay should be stopped once the untreated wells have reached confluence.

8. Centrifuge at 900g for 5 mins and remove the supernatant. Wash 3× in 500 µL blocking buffer.
9. Resuspend pellet in 500 µL blocking buffer + 1.0 µg Alexa fluor 488 labeled goat anti-mouse IgG (H+L) antibody. Incubate for 1 hr at room temperature in the dark. Mix gently every ~ 10 mins.
10. Centrifuge at 900g for 5 mins and remove the supernatant. Wash 3× in 500 µL blocking buffer.
11. Resuspend in 500 µL PBS + 2 mg RNase A + 200 ng propidium iodide (PI). Incubate at 37°C for 1 hr.
12. Analyze cells by FACS. Be sure to use appropriate doublet discrimination settings. Plot Alexa 488 (FL1-H) on a log scale across the y-axis versus PI on a linear scale on the x-axis. Mitotic cells will have a 4N DNA and should stain an order of magnitude higher for Alexa 488.

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Figure 1. Selective kinase inhibition through engineered steric complementarity

(A) The gatekeeper residue within your favorite kinase is mutated to a smaller residue to enlarge the catalytic pocket. (B) Selective inhibition is achieved amongst all other cellular kinases using the PP1 analogs 1-NA-PP1, 1-NM-PP1 or 3-MB-PP1. (C) Clustal alignment showing the hinge region and gatekeeper residue for Src and a selection of human and yeast mitotic kinases. (D) Steric complementarity visualized by overlaying the Hck kinase structure and the PP1 (brown), 1-NA-PP1 (green), 1-NM-PP1 (pink), and 3-MB-PP1 (yellow) inhibitors. The gatekeeper region is displayed in white. Adapted from (6).

Figure 2.

Key steps for generating a chemical genetic system in human somatic cells.

Figure 3. Construction of analog-sensitive kinase alleles via Gibson assembly

(A) Overlapping N and C terminal kinase fragments are generated using PCR. The tails of the forward N-terminal primer and the reverse C-terminal primer need ~20 bp of overlap homology overlap with the expression vector. The N-terminal reverse primer and the C terminal forward primer also overlap and incorporate the desired gatekeeper mutation (*).

(B) The kinase fragments are combined with the linearized expression plasmid in the Gibson assembly reaction mix, which contains three enzymatic activities: (1) an exonuclease that generates single-stranded 3' overhangs, allowing for strand annealing between the fragments; (2) a DNA polymerase that fills in any remaining gaps; and (3) a DNA ligase that seals nicks to create covalently closed circular DNA, generating the final expression construct.

Figure 4. Visualizing analog-dependent and allele-specific growth inhibition using crystal violet staining

Plk1^{wt} or Plk1^{as} cells were cultured in the presence of 1-NM-PP1 or 3-MB-PP1 (left to right: 0, 0.078 μ M, 0.313 μ M, 1.25 μ M, 5.0 μ M, and 20.0 μ M) for 8 days. Adapted from (20).

Table 1

Comparison of Methods for Probing Kinase Function

Method of kinase inactivation	Kinetics	Specificity	Penetrance
<i>RNA interference (RNAi)</i>	Delayed (2–5 days).	High potential for off target effects.	Varies depending on gene and target sequence.
<i>Dominant negative (DN)</i>	Delayed (12–48 hours).	Potential for off-target effects. Requires high levels of exogenous protein expression.	Varies with each kinase. Often no phenotype reported.
<i>Stable knockout (sKO)</i>	Stable phenotype. Not compatible with essential genes.	Highly selective.	Highly penetrant. Potential for adaptation.
<i>Conditional knockout (cKO)</i>	Delayed (2–5 days for Cre recombinase dependent excision).	Highly selective.	Highly penetrant.
<i>Pharmacological inhibitors</i>	Rapid onset (minutes – hours).	Variable Very difficult to exclude off-target effects.	Varies with each chemical. Many highly and weakly penetrant examples.
<i>Chemical Genetics</i>	Rapid onset (minutes – hours).	Highly selective (built in control for specificity).	Many highly penetrant examples.