Development of enzyme activity assays for high-throughput screening (HTS)

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1. Is the enzyme of interest because of its likely importance in a pathogen (metabolic role? genetic/chemical validation? expression data?), druggability, uniqueness relative to human enzymes, etc.?

2. What is the enzyme's assayability? That is, are its substrates readily obtainable at a reasonable cost? Does it consume a HTS-detectable substrate, and/or can it produce a HTS-detectable product (either directly or with coupling enzymes)? Review the literature to see what others have tried, either for the enzyme you're studying or for isoforms from other species. Find and/or order reagents, including buffer and product (for making standard curves).

3. Is the enzyme active? You can test this with a "low-throughput" instrument such as a UV/visible spectrophotometer, if convenient. Do a control with a "random" enzyme to make sure that the activity is due to the recombinantly expressed enzyme of interest rather than a contaminant from the expression system. Also do controls with one substrate but not the other, both substrates but no enzyme, etc. What is the enzyme's specific activity at saturating substrate concentrations? How does this compare to literature values?

4. What are the enzyme's approximate K_m's for its substrates? You can get these numbers from "low-throughput" assays, if convenient. How do they compare to literature values? K_m estimates allow you to set HTS assay concentrations can be set to ~3X each K_m, assuming that this doesn't interfere with the detection strategy. When estimating K_m's, make sure that [substrate] is well in excess of [enzyme].

5. Try the assay in HTS format (96- or 384-well plate). Some questions to consider:
   - What buffer conditions maximize enzyme activity and stability? Consider variations in [salt], [glycerol], [detergent], [BSA], pH, etc.
   - Is the reaction rate roughly proportional to enzyme concentration? When testing for possible inhibition of the enzyme, 50% inhibition (for example) is equivalent to reducing the enzyme concentration by 50%. Thus it's important to see that specific reductions in enzyme concentration lead to equivalent reductions in reaction rate.
   - How stable are the enzyme, other reagents, and readout of the enzyme's activity? Must enzyme stocks be used as soon as they are thawed? Must any reagents be made fresh each day? Must plates be read right away?
   - How does the enzyme perform when stored in 50% glycerol (to avoid freeze/thaw cycles)?
   - Will the screen be performed in "kinetic" or "end-point" mode, and how long will the reaction incubation last? Enzyme activity should stay relatively constant throughout this period.
   - What known (control) inhibitors can be used for assay validation? What are their IC_{50}s?
   - What Z-factors (Zhang et al. 1999) can be achieved in comparing wells with and without enzyme, or with and without inhibitor? These should regularly exceed 0.5.

6. As a check of your protocol, see whether a coworker can perform the assay using the directions that you have prepared and obtain data similar to yours.

7. Troubleshooting

A. *Reagent stocks*

- Are you using the intended ingredients? (For example, do you need the reduced form of NAD, or the oxidized form? Is your enzyme adenylosuccinate lyase or adenylosuccinate synthetase? Does the label on the container make it clear what's inside?)
- Is this particular batch/variety of the reagent known to give good results, or have we not tried it until now? (Example: switching from yeast pyrophosphatase to *E. coli* pyrophosphatase.)
- Is the reagent degraded? (Has it been left out overnight at room temperature when it needs to be stored in the fridge or freezer? Does Sigma package it with a special stabilizing gas that leaks out when you open the vial for the first time?)
- Is the concentration of the stock appropriate for this experiment? (Again, what does the label say? Can you trust the person who made the stock?)

B. *Procedural issues*

- If you haven't done this experiment successfully before, who has done it before?
  - Someone in the lab -- check with them directly.
  - Someone outside the lab -- check the published protocol/notes.
  - No one – good luck…. Remember that some recombinant enzymes may simply be inactive.
- If you have done this experiment successfully before, what are the differences between the previous experiment and this one?
  - Are the concentrations and volumes right? (Are concentrations listed those of the stocks, or are they the "final concentrations" once everything has been mixed together?)
  - If you're using a kit, are you completely following the kit's directions? (Example: the malachite green kit for Pi detection requires the use of buffers and reagents not exposed to Pi-containing glassware etc. And it doesn't work if you add more of Reagents A and B than recommended!) Are you mixing components as thoroughly as needed? Are your incubation times adequate? Have you added reagents in the right sequence?
  - Are you in the linear range of your detection modality? (Should enough product be present in the sample to distinguish it from a negative control? Conversely, might your signal be saturating your instrument? Beware of absorbances above 1.0!)

C. *Next steps*

- Are you not sure exactly what you just did? If so, repeat the assay more carefully, taking detailed notes.
- If studying a multistep reaction (e.g., by using coupling enzymes to detect a product), consider breaking the reaction down into individual steps to see which part is problematic. Example: in pathway A => B => C => D, first start with compound C and see if you can produce and detect D. If that works, then start with B and see if you can still get to D….

- Did the assay "just barely" work before? If so, the fact that it's not working now is unsurprising. Can the protocol be improved? Or should it be abandoned altogether?
- If you can't decide exactly what went wrong, try to identify the most likely culprit and design a simple experiment with appropriate controls to determine whether your suspicions are correct.
- If you are having trouble detecting a product of a reaction, do you have the option of detecting another product, or detecting consumption of a substrate?

REFERENCES

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