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A Screening Protocol for Identification of Functional Mutants of RNA Editing Adenosine Deaminases

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Abstract

Genetic screens can be used to evaluate a spectrum of mutations and thereby infer the function of particular residues within a protein. The Adenosine Deaminase Acting on RNA (ADAR) family of RNA editing enzymes selectively deaminate adenosines (A) in double helical RNA, generating inosine (I). The protocol described here exploits ADAR2's editing activity in a yeast based screen by inserting an editing substrate sequence with a stop codon incorporated at the editing site upstream from sequence coding the reporter α -galactosidase. A-to-I editing changes the stop codon to one for tryptophan, allowing normal expression of the reporter. This technique is particularly well-suited for screening ADAR and ADAR substrate mutant libraries for editing activity.

Keywords

RNA editing; yeast; screen; A-to-I editing; ADAR substrate

INTRODUCTION

This article describes a detailed protocol for yeast-based screens that detect functional A-to-I editing by the Adenosine Deaminase Acting on RNA (ADAR) family of RNA editing enzymes. ADARs bind to duplex RNA and deaminate adenosines (A) producing inosine (I), which is read as guanosine (G) during translation (Figure 1). This can result in amino acid substitutions, destabilization or stabilization of RNA secondary structures, alter pre-mRNA splicing patterns, and may modulate miRNA activity (Hundley and Bass, 2010). There are several different methods for detecting and quantifying editing at specific sites in cellular RNAs, including direct sequencing of cDNA products arising from reverse-transcription polymerase chain reaction (RT PCR) (Iwamoto and Kato, 2003; Vissel et al., 2001). Indeed, the different methods available for assessing editing efficiency at different sites have been reviewed (Nakae et al., 2008). The assay described here couples an editing event to the expression of the reporter α -galactosidase, a secreted enzyme from *Saccharomyces cerevisiae*. A known ADAR substrate is inserted upstream of the α -galactosidase sequence within a plasmid and the edited site on the substrate is modified to encode an in-frame stop codon. The plasmid will not express α -galactosidase unless, following transcription, the

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Internet Resources

<http://www.microbelibrary.org/component/resource/laboratory-test/3152-transformation-of-escherichia-coli-made-competent-by-calcium-chloride-protocol>

American Society for Microbiology Microbe Library. This contains a detailed protocol for the creation and transformation of chemically competent *Escherichia coli*. <http://www.yeastgenome.org/>

Saccharomyces Genome Database (SGD). The SGD is a large database of biological information on *S. cerevisiae*, including a genome browser, lists of yeast strains, additional protocols, and more.

editing site is targeted by ADAR, changing the UAG stop codon to a UGG tryptophan codon and thereby permitting translation to proceed. *S. cerevisiae* does not have ADAR or ADAR-like genes yet are able to express active ADARs from expression plasmids. *Escherichia coli* also does not have ADAR-like genes but functional ADARs have not been expressed in bacteria. If the reporter plasmid is transformed into *S. cerevisiae* expressing the ADAR, the level of α -galactosidase expression becomes a function of the level of editing at the stop codon. Expression of α -galactosidase can be detected by growing the cells in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl α -D-galactopyranoside (X- α -gal) as yeast colonies secreting α -galactosidase will turn green in its presence (Rupp, 2002). Thus green coloration indicates the presence of active ADAR while a lack of color, i.e. white colonies, indicates inactive ADAR. Since our original report of assessing ADAR activity using galactosidase activity in yeast, others have similarly coupled editing to reporter enzyme/protein expression using luciferase and green fluorescent protein (Gommans et al., 2010; Jepson et al., 2012).

A major advantage of the assay described here is that it is capable of screening large mutant libraries. ADAR activity assays typically employ RT-PCR to generate cDNA from edited RNA substrates followed by sequencing or primer extension (Roberson and Rosenthal, 2006) to detect A to G changes – costly and time-consuming when working with many different mutants. This method can screen ADAR mutants in plasmid libraries generated via saturation mutagenesis, facilitating structural studies. Optionally, a library can be constructed from the reporter plasmid itself, randomizing the sequence within the ADAR substrate. This plasmid library can be co-transformed into yeast with wild type ADAR and used to screen for functional variants of the substrate. Because this assay is qualitative, follow-up experiments are required to characterize mutations.

STRATEGIC PLANNING

Creation of Mutant Libraries

Although this technique can be used to (qualitatively) assess the editing efficacy of specific ADARs at specific sites it is most effectively employed to screen libraries of ADAR or ADAR substrate mutants, identifying active and inactive variants. ADAR libraries can be generated using site-directed saturation mutagenesis (Sanchis et al., 2008; Steffens and Williams, 2007), employing primers with randomized nucleotides at sites of interest to amplify the plasmid via PCR. It is not recommended to incorporate more than nine randomized nucleotides as the resulting plasmid library may not include all possible sequences (see below). Furthermore, an excess of randomized nucleotides will lower the melting temperature (T_m) of the primers, impeding PCR. Randomized nucleotides should be flanked by 10–15 correct bases on either side to permit specific annealing (Georgescu et al., 2003). It is recommended to randomize codons by replacing them with “NNS” where N is any natural nucleotide and S is either guanosine or cytosine as this set will encompass every possible amino acid and exclude two stop codons. The plasmid used should encode an antibiotic resistance marker (e.g. ampicillin) in order to select for transformed *E. coli*. If constructing a library of ADAR substrates using the reporter plasmid (see below), randomize nucleotides of interest while avoiding randomizing sites that could produce stop or start codons in the reading frame, if possible. Do not randomize any of the three nucleotides of the stop codon at the editing site.

Construction of Reporter Plasmid

A key feature of the screen is the insertion of an ADAR substrate into the α -galactosidase reporter. α -Galactosidase, also called melibiase or α -D-galactoside galactohydrolase, is encoded by the *MEL1* gene, approximately 1.3 kbp in length. It is used by yeast primarily to

break down the disaccharide melibiose into its component sugars, glucose and galactose. Though α -galactosidase is associated with the cell wall it will gradually secrete into its surroundings. Thus X- α -gal around yeast expressing α -galactosidase will be digested, resulting in green coloration. This property makes the reporter well-suited for plate assays (Rupp, 2002). The ADAR substrate is subjected to site-directed mutagenesis, generating a stop codon (UGA or UAG) that incorporates the edited adenosine in the same reading frame as the α -galactosidase gene; an example reporter employing the human glutamate receptor-B (GluR/B) ADAR2 editing site as the substrate is depicted in Figure 2. The introduction of the stop codon results in premature termination of translation of α -galactosidase protein, rendering it nonfunctional. In the presence of active ADAR, the A within the stop codon is deaminated, generating I. Because I is read during translation as G, the stop codon is effectively converted to one for tryptophan (UGG). This permits read-through of the complete α -galactosidase ORF. Thus the level of α -galactosidase expression is positively correlated with the level of A-to-I editing at the stop codon.

When designing a reporter plasmid it is helpful to identify and insert the minimum sequence around the edited adenosine necessary to form a functional ADAR substrate. A long polypeptide sequence encoded by an extended ADAR substrate could alter the function of the reporter. Furthermore, long ADAR substrates have a greater chance of encoding stop or start codons and forming additional secondary structures following transcription, disrupting expression. The length of the duplex and/or loop encompassing an editing site can generally be reduced while maintaining the substrate's viability as a target for ADAR (Pokharel and Beal, 2006). Ideally, the edited adenosine is highly edited within the native substrate, making it easier to identify and quantify editing in deamination assays following the screen. It is critical to ensure that the stop codon is in the same reading frame as the α -galactosidase reporter and that there are no additional in frame stop codons within the substrate. It may also be helpful to remove any methionine codons in the substrate sequence downstream of the stop codon (Gommans et al., 2010). Bear in mind that any modifications to the substrate – particularly to the region adjoining the edited adenosine – may affect ADAR binding and/or editing. Finding a balance between necessary modifications and preserving the native sequence of the substrate is crucial to the effectiveness of the reporter plasmid. It is helpful to construct several candidate reporters, each bearing different modifications, and test them as described below.

As with ADAR encoding plasmids, the reporter plasmid should include an antibiotic resistance marker to select for transformed *E. coli*. The α -galactosidase gene can be inserted into the plasmid using standard techniques. This should be followed by insertion of the ADAR substrate into the α -galactosidase gene within the plasmid incorporating the stop codon. The ADAR substrate should be inserted upstream of the α -galactosidase enzymatic domain but downstream of the secretion signal and start codon (Pokharel and Beal, 2006). It will likely be necessary to generate restriction sites in the α -galactosidase gene and the ends of the ADAR substrate via site-directed mutagenesis to facilitate the second insertion.

Once the reporter plasmid(s) is constructed and its sequence confirmed its activity should be tested. This can be accomplished by co-transforming the reporter into a strain of yeast with a plasmid expressing wild type ADAR and another strain with a plasmid expressing an inactive form of the same ADAR (e.g. E396A human ADAR2). Plate the two strains onto inducing X- α -gal media as described below. If strong green coloration is observed in the strain with wild type ADAR and no coloration is observed in the inactive strain the reporter is functioning as designed. Editing efficacy of the substrate should also be determined using an *in vitro* deamination assay employing Sanger sequencing (Nakae et al., 2008).

SCREEN FOR FUNCTIONAL ADENOSINE TO INOSINE EDITING IN YEAST BY α -GALACTOSIDASE EXPRESSION

Any strain of *S. cerevisiae* used in this screen should be chemically competent and fast-growing. Its genotype should feature the selection markers for both the reporter plasmid and the plasmid encoding ADAR. In addition, it should not transcribe the gene *MEL1* as it encodes α -galactosidase (see above); S288c and W303 strains do not transcribe *MEL1* (Rupp, 2002). The diploid strain INVSc1 (Invitrogen), genotype *MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52*, has been used with success in this screen (Pokharel and Beal, 2006; Pokharel et al., 2009). After the first successful transformation has been confirmed via growth on selective media it may be prudent to preserve this strain in glycerol stock (Fink, 2002) as it can be used again in future screens. After the second transformation, i.e., transformation with the plasmid library, the yeast should be plated on non-inducing selective media as expression of active ADAR slows yeast cell growth (unpublished observation). Once colonies have appeared, replica plating can be used to transfer them to inducing selective X- α -gal media. The X- α -gal can be mixed directly into the molten media or spread on the surface. If the latter approach is used, it is important to ensure the X- α -gal is evenly distributed across the surface of the medium. If the former approach is used, autoclave the media first and do not add X- α -gal until the molten media has cooled to approximately 55 °C. In either method, use X- α -gal media as soon as possible as X- α -gal is both light and temperature sensitive. If necessary, X- α -gal plates can be stored for short periods in the dark at 4 °C. Assuming there is A-to-I editing, green coloration should appear after approximately 72 hours. However, after an incubation period of a week or longer even cells lacking any A-to-I editing activity may exhibit weak green coloration. Thus it is critical to monitor replica plates daily to distinguish true A-to-I editing activity from false positives.

Plasmids can be isolated from yeast using a modified bacterial miniprep and sequenced using primers specific to the plasmid of interest. Because the yeast initially contain both the ADAR and reporter plasmids, it is necessary to separate them for follow-up analysis. Transform the plasmid mixture back into yeast and grow on media that will select for the desired plasmid (e.g. –ura or –trp). Use one or more of the resulting colonies in the modified miniprep as described above to isolate the plasmid of interest from the yeast. Optionally, if the reporter and ADAR plasmids use different antibiotic resistance markers the plasmids could be transformed into bacteria and grown in media with the appropriate antibiotic to select for the desired plasmid followed by bacterial miniprep.

Screened mutants can be further analyzed using *in vitro* deamination assays. Overexpress and purify ADAR in a yeast expression system (Macbeth and Bass, 2007) and incubate with appropriate RNA substrates followed by RT-PCR and sequencing or primer extension (Roberson and Rosenthal, 2006; Schirle et al., 2010) to evaluate editing at each site.

Materials—Note: This protocol assumes the reporter and ADAR plasmids encode the *trp* and *ura3* selection markers, respectively, and that are both galactose-induced and encode ampicillin resistance. Different plasmids may require different media for selection and/or induction of expression.

Wild type ADAR plasmid, 50 ng/ μ L

Reporter plasmid, 50 ng/ μ L

Phusion® High-Fidelity DNA Polymerase, 2.0 units/ μ L (New England Biolabs, cat. no. M0530S)

Phusion HF Reaction Buffer 5X (New England Biolabs, cat. no. M0530S)
dNTP mix, 10 mM
Forward and reverse primers containing randomized nucleotides
Thermal cycler suitable for PCR
Restriction enzyme DpnI, 20 units/ μ L
Chemically competent *E. coli*
Luria-Bertani + ampicillin (LB + amp) media, liquid and agar plate (see recipe)
Cell incubator with shaker
QIAprep Spin Miniprep Kit (QIAGEN, cat. no. 27104)
Chemically competent diploid *S. cerevisiae* with the appropriate selection marker genotype
YPD media, liquid and agar plate (see recipe)
YPAD media, liquid (see recipe)
Salmon sperm ssDNA, 10 mg/mL
Lithium acetate solution (see recipe)
40% PEG solution (see recipe)
Complete media (CM) –ura +glu, liquid and agar plate (see recipe)
Complete media (CM) –ura –W +glu, agar plate (see recipe)
Complete media (CM) –ura –W +gal, agar plate (see recipe)
5-bromo-4-chloro-3-indolyl α -D-galactopyranoside (X- α -gal)
Acid washed silica or zirconium beads
ADAR (or reporter) specific primers for Sanger sequencing

Generation of plasmid library—Note: Use sterile equipment, supplies, and reagents at all steps.

- 1 Perform site-directed saturation mutagenesis PCR using the ADAR or ADAR substrate primers containing randomized nucleotides with the wild type ADAR or reporter plasmid as the template, respectively.

Below are a suggested reaction mixture and time course for the PCR; alternate mixtures, reagents, and time courses may be used if preferred. It is recommended a negative control lacking polymerase be run to confirm that the template is completely digested following DpnI treatment (see below).

32.5 μ L Milli-Q water
10.0 μ L 5X Phusion HF Reaction Buffer (New England Biolabs, cat. no. M0530S)
1.0 μ L 10 mM dNTP
2.0 μ L 25 μ M forward primer
2.0 μ L 25 μ M reverse primer

2.0 μ L 50 ng/ μ L template plasmid

0.5 μ L 2.0 units/ μ L Phusion® High-Fidelity DNA Polymerase (New England Biolabs, cat. no. M0530S)

Initial Denaturation, 1 cycle

30 seconds, 98 °C

Amplification, 30 cycles

Denaturation, 10 seconds, 98 °C

Annealing, 30 seconds, 65 °C

Extension, 60 seconds per 1 kbp length of plasmid, 72 °C

Final Extension, 1 cycle

7 minutes, 72 °C

Hold

4 °C

- 2 Add 1 μ L 20 units/ μ L DpnI per 50 μ L PCR reaction and incubate 1 hour at 37 °C to digest template plasmid. Heat inactivate DpnI by incubating for 20 minutes at 80 °C.

This step ensures only plasmids generated from the randomized primers remain.

- 3 Transform chemically competent *E. coli* with DpnI-treated PCR reaction mixture. Plate cells on LB + amp media overnight at 37 °C

- 4 Combine all colonies on the plate into a single 5 mL LB + amp culture. Incubate overnight in shaker at 300 rpm at 37 °C.

The more randomized sites present in a library the greater number of colonies the library must be drawn from to ensure it encompasses every possible combination of nucleotides. A library with a single randomized codon, NNS, should be created from least 72 colonies, while a library with three randomized nucleotides, NNN, should be generated by combining at least 144 colonies (Georgescu et al., 2003). Libraries with two or three randomized codons (or 4 or more randomized bases) demand thousands of colonies; it will be necessary to prepare multiple plates from the same transformation when generating libraries with this many randomized sites.

- 5 Extract the plasmid library from the combined *E. coli* culture using the QIAprep Spin Miniprep Kit or equivalent method.

Transform yeast with reporter or wild type ADAR plasmid (lithium acetate transformation)—Note: Alternate lithium acetate transformation protocol(s) may be used, if preferred.

- 6 Streak yeast cells across YPD plate and incubate at 30 °C. When colonies appear, take one and place in 5.0 mL YPD. Incubate 17 hours in shaker at 300 rpm at 30 °C.

It isn't critical that the cells be incubated for exactly 17 hours, but an extended incubation may reduce the transformation efficiency.

- 7 Add 1 mL of yeast culture in YPD from previous step (6) to 10 mL YPAD. Incubate 5 hours in shaker at 300 rpm at 30 °C.

Ideally, the final concentration of cells in the culture should be 2×10^7 cells/mL. The cell concentration can be estimated using a spectrophotometer by measuring its optical density (OD) at 600 nm; an OD₆₀₀ of 1.0 corresponds to a concentration of approximately 1.0×10^6 cells/mL.

- 8 Pellet yeast YPAD culture from previous step (7). Discard the supernatant and re-suspend cells in 5 mL lithium acetate solution. Pellet cells again. Discard most of the supernatant, leaving ~250 µL behind. Re-suspend cells in the remaining supernatant. Take 7 µL of 10 mg/mL salmon sperm ssDNA in microcentrifuge tube, incubate 10 minutes at 95 °C and then let sit at room temperature. Add 4 µL of 50 ng/µL plasmid to heated salmon sperm ssDNA, gently mix via pipetting, and add 60 µL of the re-suspended yeast and mix. Add 450 µL 40% PEG solution and gently mix. Incubate yeast 30 minutes at 30 °C.

Higher plasmid concentrations yield greater transformation efficiency, but do not use more than 5 µg total plasmid in a single transformation. Start incubation of the salmon sperm ssDNA before centrifuging the yeast culture to save time. Be sure to add the plasmid DNA to the ssDNA before adding the cell lithium acetate suspension. The 30 °C incubation can be extended to 40 minutes without affecting the transformation efficiency.

- 9 Heat shock yeast for 15 minutes at 42 °C and then let sit for 2 minutes at room temperature.
- 10 Spread 150 µL of the yeast solution on solid CM –ura +glu or CM –W +glu depending on whether an ADAR or reporter plasmid, respectively, was transformed into the yeast. Incubate at 30 °C.

It is advisable to let the cultures sit with the solid media on the bottom of the plate for a time to allow the PEG/yeast solution be fully absorbed into the media. This helps ensure the yeast is evenly spread across the surface and does not pool.

Transform reporter yeast with ADAR or ADAR substrate plasmid library (lithium acetate transformation)

- 11 Once colonies appear, transfer one colony to 10 mL CM –ura +glu or CM –W +glu depending on whether the yeast contains an ADAR or reporter plasmid, respectively. Incubate for 17 hours in shaker at 300 rpm at 30 °C.
- 12 Add 1 mL of yeast culture in CM –ura +glu or CM –W +glu from previous step (11) to 10 mL YPAD. Incubate 5 hours in shaker at 300 rpm at 30 °C.
- 13 Pellet yeast YPAD culture from previous step (12). Discard the supernatant and re-suspend cells in 5 mL lithium acetate solution. Pellet cells again. Discard most of the supernatant, leaving ~250 µL behind. Re-suspend cells in the remaining supernatant. Take 7 µL of 10 mg/mL salmon sperm ssDNA in microcentrifuge tube, incubate 10 minutes at 95 °C and then let sit at room temperature. Add 4 µL of plasmid library to heated salmon sperm ssDNA, gently mix via pipetting, and add 60 µL of the re-suspended yeast and mix. Add 450 µL 40% PEG solution and gently mix. Incubate yeast 30 minutes at 30 °C.

- 14 Plate 150 μ L of the yeast solution on solid CM –ura –W + glu. Incubate until colonies appear at 30 °C.

Replica plate yeast colonies onto expression inducing X- α -gal medium

- 15 Make a replica plate of each of the CM –ura –W + glu yeast plates from the previous step (9) using CM –ura –W + gal + X- α -gal plates. Incubate at 30 °C and monitor colonies for green coloration.

Even more so than in step 10 it is important to ensure the cells are evenly spread on the plate at this step as pooling may result in tight clusters of cells, making it difficult to pick individual colonies. It may also lead to false positives and/or false negatives if the X- α -gal is not evenly distributed throughout the media.

Isolate plasmids from yeast colonies

- 16 Transfer colony to 5 mL CM –ura –W + glu. Incubate 48 hours in shaker at 300 rpm at 30 °C.
- 17 Pellet yeast culture and re-suspend cells in 250 μ L P1 buffer from QIAprep Spin Miniprep Kit. Add 100 μ L acid-washed silica or zirconium (glass) beads.

To measure the volume of acid-washed glass beads, take a microcentrifuge tube or similar container and add 100 μ L water. Take another tube and slowly pour the glass beads into it until they are at the same level as the water in the other tube.

- 18 Vortex the cells for 10 minutes on highest speed.
- 19 Transfer the yeast lysate to different container. Add 250 μ L P2 buffer from QIAprep Spin Miniprep Kit. Let sit for 5 minutes at room temperature.
- 20 Add 350 μ L N3 buffer from QIAprep Spin Miniprep Kit and shake by inverting 10 times.
- 21 Proceed with purification of plasmid as though the yeast lysate were bacterial lysate, following the protocol for plasmid DNA purification using the QIAprep Spin Miniprep Kit included in the QIAprep Spin Miniprep Kit.

The key difference between this protocol and a traditional bacterial miniprep is the addition of acid-washed glass beads and the 10 minute vortex. This is necessary in order to fracture the yeast cell walls. Afterwards it is no different from the protocol provided by the manufacturer.

- 22 Transform the purified plasmids into yeast following steps 6–10, growing the yeast on solid media that will select for the mutant plasmid.
- 23 Repeat steps 16–21 to isolate the mutant plasmid from the yeast colonies.

Mutant ADARs can be overexpressed and purified in a yeast expression system (Macbeth and Bass, 2007) while ADAR substrates can be transcribed via in vitro transcription. Both products can then be used in deamination assays to further characterize the effects of the mutations (Schirle et al., 2010).

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps.

YPD

10.0 g yeast extract
20.0 g peptone
20.0 g agar (if making solid media)
Raise volume to 900 mL with water and autoclave
Add 100 mL filter-sterilized 20% glucose (w/v); final volume should be 1 L
Pour media into plates if making solid media
Store until discoloration/contamination occurs at 4 °C

YPAD

As YPD above, but add 100 mg adenine hemisulfate prior to autoclave

Lithium acetate solution

10 mM Tris-HCl pH 7.5
1 mM EDTA (use stock solution of pH of 8.0)
100 mM Lithium Acetate
Store indefinitely at room temperature

40% polyethylene glycol (PEG) solution

As lithium acetate solution above, but 40% PEG 4000 (w/v)

Complete (CM) media for yeast

6.7 g yeast nitrogen base without amino acids
22 mg adenine hemisulfate
1.92 g of appropriate yeast synthetic dropout medium supplement*
15 g agar (if making solid media)
Raise volume to 900 mL with water and autoclave
Add 100 mL filter-sterilized 20% glucose or filter-sterilized 30% galactose (w/v)
Add 1 mL 5-bromo-4-chloro-3-indolyl α -D-galactopyranoside (X- α -gal) if making replica plates with X- α -gal incorporated into the media
Final volume should be 1 L
Pour media into plates if making solid media
If making replica plates with X- α -gal on the surface, spread 200 μ L X- α -gal once the media has solidified. To help ensure the surface is evenly coated, add a quantity of acid-washed glass beads sufficient to cover $\frac{1}{4}$ the media. Gently shake the plate and let it dry in a dark place.
Store until discoloration/contamination occurs at 4 °C. Plates containing X- α -gal should be used immediately

*Mass required for 1 L media may vary depending on the supplement; follow the manufacturer's instructions

5-bromo-4-chloro-3-indolyl α -D-galactopyranoside (X- α -gal)

0.1 mg/mL final concentration, stock solution 6 mg/mL in DMF

COMMENTARY**Background Information**

ADAR proteins bind to duplex RNA via N-terminal double-stranded RNA binding motifs (dsRBMs) while the deamination takes place at C-terminal catalytic domains. Although there are sequence and structural preferences (Bass, 1997) which can result in near complete A-to-I editing at some sites, low-level nonspecific editing can occur -particularly in long, perfectly base-paired duplex RNA (Eggington et al., 2011). Examples of ADAR substrates include transcripts for the human glutamate receptor B, 5, and 6 subunits, serotonin receptor 2C subtype, Kv1.1 potassium ion channel, NEIL1 DNA repair glycosylase and ADAR2 itself (Maydanovych and Beal, 2006; Yeo et al., 2010). Owing to the chemical similarity between inosine and guanosine, the only difference being an amine at the C2 position, I is read as G by the ribosome. This can cause codon changes, potentially resulting in amino-acid substitutions within the ORF of a protein.

As previously mentioned, the primary advantage the protocol described here its ability to screen mutant libraries of ADAR or ADAR substrates for editing. An alternate approach would be to generate mutant ADARs or ADAR substrates via site-directed mutagenesis and performing *in vitro* deamination assays to determine the level of editing for each ADAR and/or ADAR substrate. While such an approach would yield quantitative data it is not ideal for assessing the role multiple amino acids and/or nucleotide positions. Such studies would require performing dozens or even hundreds of separate deamination assays. This screen selects for deamination activity *in vivo*, facilitating the identification of active ADAR or ADAR substrate variants from a large pool.

Critical Parameters

Reporter plasmid design and selection—It is essential to sequence and test the reporter plasmid before using it in a screen and/or using it to generate an ADAR substrate library. Ensure that the template plasmid is compatible with *S. cerevisiae* and encodes a marker that will select for transformed yeast.

Plasmid library composition—When generating plasmid libraries it is advisable to generate and combine as many transformed bacterial colonies as possible to maximize the diversity of the library. Should any of the original template plasmid remain in the reaction mixture following saturation mutagenesis PCR to generate a mutant library, some or all of the bacterial colonies that appear following transformation may contain the template rather than mutated plasmids. This will reduce the library's diversity. The restriction enzyme DpnI will cleave methylated GATC sites, thus selectively digesting plasmids derived from bacteria while leaving plasmids generated via PCR (i.e. plasmids derived from randomized primers) intact. Therefore, saturation mutagenesis PCR products should be treated with DpnI before being used to transform *E. coli*. Be certain to run a negative control PCR lacking polymerase. Following treatment with DpnI and transformation, no bacterial colonies should grow in the plate transformed with the negative control PCR product.

Transformation of yeast and colorimetric screen—If screening ADAR mutants, be sure to transform the reporter plasmid followed by the ADAR library. If screening ADAR substrate mutants, be sure to transform wild type ADAR followed by the ADAR substrate library. Because very low levels of X- α -gal could result in false negatives while an excess of the chromogenic substrate could result in false positives it is very important that the X- α -

gal – whether it is spread on the surface of the medium or directly incorporated into it – be homogenously distributed throughout the media. If X- α -gal is added to the surface of solid media it is advisable to use glass beads to ensure it is evenly spread as described above. Note that weak green coloration may appear even in colonies with no A-to-I activity after an incubation period of a week or longer. Although it is usually possible to distinguish true positives by the intensity of the color under these circumstances it is advisable to maintain a daily photographic record of the replica plates.

Troubleshooting

Generating plasmid libraries—If colonies appear on LB + amp plates containing *E. coli* transformed with the negative control PCR product (i.e. no polymerase) it is likely the template was not completely digested by DpnI. Rerun the PCR and extend the DpnI digest by an hour and/or use new DpnI. If few (<100) or no bacterial colonies appear on the other plates, the strain of *E. coli* used may be weakly competent. Make or purchase new ultracompetent cells and try again.

Colorimetric yeast colony screen—A complete lack of green colonies on a replica plate could simply be because there are no active mutants present on the plate. Recreating the plasmid library from a greater number of bacterial colonies and repeating the experiment may result in active mutants. However, it is also possible the plate's X- α -gal has been compromised. One way to test for this is to prepare a positive control plate by replica plating (or streaking) yeast transformed with wild type ADAR and an active reporter onto selective X- α -gal media made using the same source of X- α -gal. If no green coloration appears in the colonies on the positive control plate, the X- α -gal has likely degraded before or during preparation of the media. Try making new X- α -gal plates or use fresh X- α -gal.

Anticipated Results

The number of yeast colonies with active ADAR or ADAR substrate (green) will vary depending upon which nucleotides in the sequence are randomized. In general, ADAR substrate libraries will yield fewer green colonies if the region adjoining the edited A is subjected to randomization (Pokharel and Beal, 2006). See Figure 3 for an example of a replica plate containing yeast transformed with an ADAR2 library and ADAR2 substrate human glutamate receptor-B (GluR/B) R/G hairpin stem. In addition, Figure 4 shows results of a screen employing an ADAR2 library that varied the identity of active site residues.

Time Considerations

It should take 3–4 days to generate ADAR or ADAR substrate libraries assuming all the reagents, including the plasmids and primers, are at hand. Lithium acetate transformation of yeast takes 4–5 days per plasmid. Green coloration should appear on replica plates approximately 3 days after the yeast colonies have been transferred. Isolating plasmids from yeast colonies described in steps 16–23 takes 7–9 days.

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Figure 1. Deamination of adenosine by ADAR, forming inosine (I). Because of its chemical similarity to guanosine (G), I is read as G by the ribosome.

Figure 2.

Example α-galactosidase reporter. a) Schematic of a reporter mRNA, indicating the α-galactosidase gene the ADAR substrate and the edited stop codon. b) Partial sequence of reporter mRNA with modified ADAR2 substrate human glutamate receptor-B (GluR/B) R/G hairpin stem and corresponding codons.

Figure 3. Example replica plate (CM –ura –W +gal + X- α -gal) with α -galactosidase secreting colonies. Yeast (INVSc1) are transformed with ADAR2 library and reporter plasmid using the GluR/B R/G hairpin stem as the substrate.

Figure 4.

The screening protocol can be used to probe the role of ADAR active site residues. Shown is a model of nucleotide binding in the ADAR2 active site derived from the x-ray crystal structure of the human ADAR2 deaminase domain along with mutations found in active enzymes from a library that simultaneously varied codon sequence for positions 375 and 455 in human ADAR2 (Macbeth et al., 2005; Pokharel et al., 2009).