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A Cell-Based High-Throughput Method for Identifying Modulators of Alternative Splicing

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Abstract

Alternative splicing, a key regulatory process of gene expression, is controlled by *trans-acting* factors that recognize *cis*-elements in premature RNA transcripts to affect spliceosome assembly and splice site choices. Extracellular stimuli and signaling cascades can converge on RNA binding splicing regulators to affect alternative splicing. Defects in splicing regulation have been associated with various human diseases, and modification of disease-causing splicing events presents great therapeutic promise. Determining splicing regulators and/or upstream modulators has been difficult with low throughput, low sensitivity, and low specificity. IRAS (identifying regulators of alternative splicing) is a novel cell-based high-throughput screening strategy designed specifically to address these challenges and has achieved high throughput, high sensitivity, and high specificity. Here, we describe the IRAS method in detail with a pair of dual-fluorescence minigene reporters that produces GFP and RFP fluorescent signals to assay the two spliced isoforms exclusively. These two complementary mini-gene reporters alter GFP/RFP output ratios in the opposite direction in response to only a true splicing change. False positives from a signal screen do not stimulate opposite changes in GFP/RFP ratios. The reporter pair in

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[1]The two reporters, when expressing in cells, should exhibit similar splicing levels of the inserted alternative exons. They should also express both GFP and RFP, but with opposite preference. Any known modulator of the inserted alternative exon should change the GFP/RFP ratio of the reporters, and more importantly in the opposite direction for the two reporters. In addition to examining the performance of the reporters, these tests also confirm a known modulator suitable as a spike-in control in the final screen.

[2]Reporter minigene expression varies substantially among stable cell clones. Cell clones of higher expression is not necessarily better, because they may not be as efficiently transfectable or responsive to known regulators. Furthermore, some clones can change GFP/RFP ratios even when transfected with an empty control vector as we have observed. Therefore, multiple clonal lines should be tested to find the optimal one.

A good cell clone does not change GFP/RFP ratio when transfected with an empty control vector, and should do so in response to transfection with a known regulator of the exon. An RT-PCR assay to measure the splicing changes at the RNA level is also recommended. A good clone should also be efficiently transfectable. After transfection with a GFP expression plasmid, a highly transfectable cell clone shows very high GFP signals without affecting the RFP signals.

[3]IRAS is broadly applicable to screen any library with considerations taken in light of the library attributes. Loss-of-function screens often require multiple shRNA or siRNA hits targeting the same gene to declare a true target. Because of functional compensation, regulatory paralogs may not be easily identified in loss-of-function screens. Loss-of-function screens may also miss true regulators that are absent in the screened cells. Gain-of-function screen has less concern of off-target effects, paralogous compensation, or missing expression, but overexpression can induce artificial splicing changes.

[4]The screening parameters and the kinetics of reporter expression need to be examined for screening different libraries or in different cell types. For example, we have seen that cDNA overexpression plateaus at 2 days posttransfection in N2a cells but RNAi depletion typically requires 3 days or longer. To avoid overconfluency within a well, the number of cells initially plated needs to be optimized; this can affect the transfection condition.

[5]To meet the similar data quality, pixel size of 100 μm should be used to screen 1536-well plates.

[6]Overlapping candidates typically constitute about 5–20% of total hits. The candidates should be tested for their actions on the splicing of the endogenous exon, because minigene reporters do not necessarily recapitulate the regulation of endogenous exons. The minigene may not contain all of the relevant *cis*-regulatory elements. Molecules that affect the splicing of the minigene reporter need to be confirmed on endogenous transcripts in the relevant cell type. This can be achieved by either overexpressing the cDNA candidate or RNAi depletion of the candidate gene followed by RT-PCR assay of the endogenous spliced variants.

conjunction with robotic liquid handlers and arrayed libraries allows IRAS to screen for both positive and negative splicing regulators with high sensitivity and specificity in a high-throughput manner.

Keywords

Dual-fluorescence; Dual-output; Minigene; Splicing reporters; High-throughput screen; Alternative splicing; GFP; RFP; Splicing regulator; IRAS

1 Introduction

Many pre-mRNA alternative splicing events are highly controlled to produce functionally distinct gene products during development or in response to extracellular stimuli [1–3]. A single alternative exon typically contains multiple *cis*-elements in itself or its surrounding introns, and is thus regulated by multiple cognate trans-factors [4, 5]. On the other hand, each *trans*-factor can affect many target exons [6]. Despite extensive studies on a dozen of RNA binding proteins that have been characterized as alternative splicing regulators in eukaryotic cells, many more await discovery to fill in the large alternative splicing regulatory network that impact almost every multi-exon gene in all aspects of biology. Factors modulating the activity of RNA binding splicing regulators are also poorly understood.

Global identification of exon targets for individual splicing factor has been aided by genome-wide sequencing-based methods [7–10]. However, identification of splicing regulators for a specific alternative exon is generally difficult and intensively laborious. Successful screens were reported isolating single splicing factor, but were mostly insensitive for broader discovery of regulatory factors [11–13]. Most of these methods used a single-output splicing reporter for which splicing changes were measured by changes in expression of one of the two isoforms. Transcriptional activity of the reporters apparently contributed to the readout of these single-output reporters, and thus led to a multitude of false positives. Additionally, the single-output reporters had to be spliced at very low basal levels to obtain a large signal-to-noise ratio for the screens. The use of a pair of single-output reporters could overcome some of these limitations [14], but was confronted with new variables resulting from unpredictable integration of two minigene reporters into genomes.

False positive hits modulating transcriptional activity in screening can be mitigated by dual-output reporters in which both splicing isoforms are assayed allowing for screening of changes in isoform ratio. Furthermore, the expression of the two alternative isoforms is negatively correlated, so using the ratio of the two isoforms to measure splicing level squares the dynamic range given by individual output. Various versions of dual-fluorescence splicing reporters have been successfully used to identify modulators of splicing [15, 16]. However, variables affecting the readout (i.e., the output ratio of the two isoforms) without an impact on pre-mRNA splicing still constituted a majority of (often about 90%) hits, probably because splicing regulation in a form of splicing ratio was typically moderate in comparison to many other gene regulation steps.

Our strategy, IRAS, uses two complementary dual-output minigene reporters that exhibit opposite changes of the readouts in response to splicing alternation (Fig. 1a). Either fluorescence-based or luminescence-based readout is compatible with IRAS. We herein describe the IRAS method using proven fluorescence-based reporters. The first reporter (pflareG) produces GFP when an exon is included and RFP when the exon is skipped, while a second reporter (pflareA) produces RFP and GFP to represent exon inclusion and exclusion respectively (Fig. 1a). Therefore, an increase in exon inclusion is reflected by an increase in the GFP/RFP ratio in the plareG reporter but a decrease in the GFP/RFP ratio in the pflareA vector. The IRAS strategy screens the same library with the two reporters in parallel. A true splicing modulator should change the GFP/RFP ratios of the two reporters in the opposite direction (Fig. 1b). For example, a splicing activator increases the GFP/RFP ratio in the pflareG reporter but decreases the GFP/RFP ratio in the pflareA reporter. A typical false positive from a single screen either preferentially stabilizes one of the fluorescent proteins (False #1 or #4 in Fig. 1a) or differentially affects ORF translation efficiency between GFP and RFP (False #2 or #3 in Fig. 1a). This false positive changing the GFP/RFP ratio of the first minigene is very unlikely to change the GFP/RFP ratio of the second minigene in the opposite direction, because pflareA and pflareG minigenes differ in only a few nucleotides. Instead, this false positive more likely changes GFP/RFP ratios of the two reporters in the same direction when affecting only either GFP or RFP ORF of the reporters or only affecting the stability of one fluorescent protein (Fig. 1b). Overlapping the screening results from these two reporters significantly eliminates false positives and enriches for identification of true splicing regulators.

The readout of pflare reporters can be detected by fluorescence imaging. Typhoon imager is preferred over conventional fluorescence microplate readers because of the confocal optics of Typhoon imager reducing background signals from media above the cells. Data points yielded from Typhoon imaging equal overall fluorescence intensity of all the cells within individual wells. Alternatively, high-content fluorescence imager produces multi-dimension data including fluorescence intensity, cell shape, size, and cell count. However, its imaging and data processing time significantly reduce the throughout.

The versatility of fluorescence imaging is enhanced by high-throughput robotic liquid-handling systems and catalogued libraries which enable screening at greater depth as well as identification of multiple regulators from a single screen. Here in detail we describe the IRAS method using a cDNA library for a gain-of-function genetic screen (Fig. 2). IRAS can also be used to screen libraries of siRNAs or shRNAs, and of small molecules, and is easily adaptable for any cassette exon of interest for genome-wide identification of its regulatory factors [17].

2 Materials

2.1 Construction of Dual-Fluorescence Minigene Reporters

- pflareA backbone reporter.
- plfareG backbone reporter.
- Phusion High-Fidelity DNA polymerase.

- Purified genomic DNA containing the gene and exon of interest.
- QuikChange II XL Site-Directed Mutagenesis Kit or similar.
- QIAquick Gel Extraction Kit or similar.
- NanoDrop.
- Alkaline phosphatase.
- T4 DNA Ligase.
- TOP10 Competent Cells.
- EcoRI.
- BamHI.
- LB-kanamycin agar plate.
- Plasmid Miniprep Kit.
- Agarose gel electrophoresis setup.

2.2 Generation of Stable Cell Clones

- A parental cell line of choice.
- QIAquick Gel Extraction Kit.
- DraIII.
- Lipofectamine 2000.
- G418 or Geneticin.
- Phenol red free media.
- FACS machine.

2.3 Library and Array Construction

- The Mammalian Gene Collection (MGC) library: developed by NIH and representing the most extensive collection of mammalian cDNA clones available. All cDNA clones are in the pCMVsport6.0 or pCMVsport6.1 backbone vectors prearrayed in 96-well plates (Life Technology).
- 384-well glass-bottom plates.
- Genetix Qbot (Molecular Devices).
- Plasmid mini-prep consumables (Macherey-Nagel).
- Biomek FX robot (Beckman Coulter).

2.4 Cell-Based High-Throughput Screens

- Trypsin.
- Report cell lines.

- Arrayed library plates.
- Lipofetamine 2000.
- Opti-MEM.
- Multidrop 384.

2.5 Data Acquisition

- Typhoon laser scanner equipped with lasers and filters for GFP and RFP (GE Healthcare Life Sciences).
- ImageQuant TL Software (GE Healthcare Life Sciences).

3 Methods

3.1 Construction of Dual-Fluorescence Minigene Reporters

The alternative exon of interest and its flanking intronic sequences are inserted into the EcoRI and BamHI sites of the pflareA or pflareG vectors. Different lengths of intronic sequences can be chosen, but conserved intronic sequence is recommended to be included because of its potential regulatory features [18]. The reporters with mid-range inclusion ratios allow screening for both positive and negative regulators simultaneously, whereas those with very high or very low inclusion ratios allow screening for only repressors or activators respectively.

1. Design forward and reverse primers for cloning the alternative exon of interest and its flanking introns.
2. Synthesize the forward primer with an EcoRI recognition site at the 5′ end. Synthesize the reverse primer with a BamHI recognition site at the 5′ end.
3. PCR amplification using the above forward and reverse primers, Phusion high-fidelity DNA polymerase, and genomic DNA as the template.
4. Confirm the PCR amplicon by agarose gel electrophoresis.
5. Excise the band of the PCR amplicon and purify the PCR product with QIAquick Gel Extraction Kit.
6. Measure the concentrations of the recovered PCR products with NanoDrop.
7. Digest the PCR product with EcoRI and BamHI simultaneously for 1 h at 37 °C. Meanwhile linearize the pflareA and pflareG vectors with EcroRI and BamHI for 1 h at 37 °C.
8. Dephosphorylate the linearized vectors with alkaline phosphatase for 0.5 h at 37 °C.
9. Confirm the linearized backbone vectors by agarose gel electrophoresis.
10. Gel-purify the digested PCR products and vectors using QIAquick Gel Extraction Kit.

11. Ligate the PCR product to the linearized vector with T4 DNA ligase at 16 °C overnight.
12. Transform the ligated products into TOP10 competent cells.
13. Plate the transformed competent cells on LB-kanamycin agar plates and incubate at 37 °C overnight.
14. Select several colonies for amplification and purification of pflareA-exon and plfareG-exon reporters using Plasmid Miniprep Kit.
15. For the plfareA-exon vector, mutate any ATG codons present within the alternative exon of interest to ATA using the QuikChange II XL Site-Directed Mutagenesis Kit.
16. For the pflareG-exon reporter, an ATG start codon within the alternative exon in frame with the GFP ORF is required for expression of GFP from exon-included transcripts. If missing, a start codon needs to be created using the QuikChange II XL Site-Directed Mutagenesis Kit. The Kozak consensus sequence gccRccATGG is also recommended to promote translation initiation for the GFP ORF.
17. Transform and purify the final versions of the pflareA-exon or pflareG-exon vectors. These vectors shall be tested for their reproducibility and applicability for the screen (*see* Note 1).

3.2 Generation of Stable Cell Clones

The choice of parental cells lines should meet the following criteria. The cell line ideally has characteristics of or is derived from the primary cells of interest. This is particularly important for loss-of-function screens because the most interesting cell/tissue type specific regulators sometimes express only in a highly relevant cell line. The cell line also needs to be highly transfectable for genetic screens to have reasonable signal-to-noise ratios.

1. Linearize the pflareA-exon vector by DraIII digestion and gel purify the linearized vector with QIAquick Gel Extraction Kit.
2. Transfect the linearized pflareA-exon vector into the target cell line of choice using Lipofectamine 2000.
3. Maintain and split the transfected cells in G418-containing media for 2 weeks to enrich stable cell clones expressing the reporter.
4. Sort double fluorescent (i.e., GFP + RFP+) single cells to individual wells of multi-well plates with a FACS machine and allow cell clones to grow and expand in G418-containing media. Some cell clones may lose GFP and RFP expression during the selection process and can be discarded. From now on, the cells need to be maintained in phenol red-free media for enhanced fluorescence visualization and imaging.
5. Test multiple cell clones for their screening feasibility (*see* Note 2).

6. Repeat steps 1–5 to generate stable cell clones expressing pflareG-exon reporter. The stable cell clones need to be confirmed for their responsiveness to splicing changes before high-throughput screens (*see* Note 2).

3.3 Library and Array Construction

1. Duplicate the MGC collection bacteria plates into 96-well plates using Genetix Qbot (Molecular Devices). This library contains about 16 thousand clones. Grow the culture overnight.
2. Prepare the plasmid DNA of the library using plasmid prep kit (Macherey-Nagel) with Biomek FX robot (Beckman Coulter).
3. Normalize plasmid concentration to 20 ng/ul for each well.
4. Spot the cDNA library into 384-well assay plates. We typically spot 40 ng cDNA (i.e., 2 μL) per 384-well (except the wells of A-H23 and A-H24).
5. Spot 40 ng negative control (pCMV sport 6.0) plasmids to wells of C23, C24, D23, and D24, 40 ng plasmids of a positive regulator (if available) to wells of E23, E24, F23, and F24, as well as a negative regulator (if available) to wells of G23, G24, H23, and H24 (16). If positive or negative control regulators are not available, leave the corresponding wells empty.
6. Two identical sets of “assay ready” library plates are prepared for pflareA and pflareG screens respectively. The assay plates take days to prepare and should be kept in −80 °C until use (*see* Note 3).

3.4 Cell-Based High-Throughput Screens

Cell number and transfection conditions shall be optimized (*see* Note 4). We have identified the optimal reverse-transfection condition for N2a cells using 8000 cells per 384-well with 40 ng plasmid DNA and 0.12 μL Lipofectamine 2000. One cDNA library screen encompassing 45384-well plates requires about 150 million cells and 2 mL Lipofetamine 2000.

1. Scale up the reporter cell cultures to ten 15 cm culture dishes by the day of performing the screens.
2. Thaw the cDNA plates while the reporter cells and transfection reagents are being prepared.
3. Dissociate the cells by trypsin.
4. Resuspend and dilute the cells in phenol-red-free feeding media to be 400 thousand cells per milliliter.
5. Mix 2 mL Lipofetamine 2000 with 166 mL Opti-MEM.
6. After the cDNA plates are thawed, dispense 10 μL Opti-MEM containing 0.12 μL Lipofectamine 2000 into each well with Multidrop 384 to mix with the plasmid DNA in the plates.

7. Dispense 8000 reporter cells in 20 μL feeding media to every well except A23, A24, B23, and B24 wells using Multidrop 384.

8. Dispense cell-free feeding media into A23, A24, B23, and B24 of all plates. These four wells will be used to derive a scale factor for inter-plate normalization (*see* below).

9. Dispense cell-free media to one (or multiple) new 384-well plate as a background plate to measure background GFP and RFP fluorescence intensity of the cell media.

3.5 Data Acquisition

1. 48 h after transfection, arrange and scan the plates on the Typhoon scanner using 200 μm pixel size as the scanning resolution for 384-well plates (*see* Note 5). GFP signals are acquired using the blue laser (488 nm) and 520 nm band pass emission filter. RFP signals are obtained using the green laser (532 nm) and 610 nm band pass emission filter. Keep the scanning parameters (including the PMT voltages) consistent across all plates.

2. Use ImageQuant TL software to analyze the scanned images (.gel files) and obtain raw GFP and RFP fluorescence intensity of every well.

3. Export the GFP and RFP signal values into Data/Text/Excel files.

3.6 Data Analysis

1. Wells A23, A24, B23, and B24 of each plate are used to derive a scaling factor for normalizing that plate. For example, XFP_{ijk} is the raw GFP or RFP intensities of a well at row "i" column "j" in plate k. Calculate the scale factor S_k for plate k based on the A23, A24, B23, and B24 wells by the following equation:

$$S_k = \frac{1}{4} \sum_{\substack{p=A,B \\ q=23,24}} \frac{\overline{XFP_{pq}}}{XFP_{pqk}}$$

where $\overline{XFP_{pq}}$ is the average values across all the plates.

2. The normalized and background-corrected GFP and RFP expression values (XFP_x) of the stable cells after transfection with cDNA x at row "i" column "j" in plate c are calculated with the following equation:

$$XFP_x = XFP_{ijc} S_c - \frac{1}{n_b} \sum_{b=1}^{n_b} XFP_{ijb} S_b$$

where S_c and S_b are the scale factors for the cell plates and the background plates respectively, and n_b is the total number of background plates used.

3. To estimate the splicing ratio of the reporter upon expression of cDNA *x*, use $\frac{GFP_x}{RFP_x}$ for the pflareG-exon minigene reporter and $\frac{RFP_x}{GFP_x}$ for the pflareA-exon minigene reporter.

4. To calculate the basal splicing level of the reporter, use the four wells (i.e., C23, C24, D23, and D24) that are transfected with empty control vector in every plate and derive the mean $\frac{GFP_{\mathrm{ctrl}}}{RFP_{\mathrm{ctrl}}}$ in the pflareG-exon cells and the mean $\frac{RFP_{\mathrm{ctrl}}}{GFP_{\mathrm{ctrl}}}$ in the pflareA-exon cells.

5. To examine the effect of cDNA *x* on the splicing of the pflareG reporter, use the following formula to approximate the change in the splicing ratio (M_x):

$$M_x = log_2\left[\frac{GFP_x}{RFP_x}\right] - log_2\left[\frac{GFP_{\mathrm{ctrl}}}{RFP_{\mathrm{ctrl}}}\right]$$

where $\frac{GFP_{\mathrm{ctrl}}}{RFP_{\mathrm{ctrl}}}$ is derived from the same plate as cDNA$_x$ Similarly, to examine the action of cDNA *x* on the splicing of the pflareA reporter, calculate the change in the splicing ratio (M_x) as followed:

$$M_x = log_2\left[\frac{RFP_x}{GFP_x}\right] - log_2\left[\frac{RFP_{\mathrm{ctrl}}}{GFP_{\mathrm{ctrl}}}\right]$$

where $\frac{RFP_{\mathrm{ctrl}}}{GFP_{\mathrm{ctrl}}}$ is derived from the same plate as cDNAx. A value of $M_x > 0$ indicates a possible increase in splicing by cDNAx. A value of $M_x < 0$ indicates a possible decrease in splicing.

6. To assess the overall fluorescence expression of cells after transfection, or the "A" value, use the following equation:

$$A_x = \frac{1}{2}\left(log_2\left(GFP_x\right) + log_2\left(RFP_x\right)\right)$$

Multiple variables, including live cell numbers, transcriptional activity and translational activity of the reporter can have an impact on *A* values.

7. The *M* values, indicative of splicing changes, can be scatter plotted against the *A* values to visualize the performance of the whole library.

8. With the R package locfdr, calculate z score and local false discovery rate (FDR) for individual cDNA by comparing their M values to the population distribution of M values. We have found that a typical FDR threshold of 0.05 has yielded relatively high sensitivity (80–88%) for our reporter screens.

9. The two parallel screens using pflareA and pflareG reporters produce two different sets of possible splicing regulators. Overlap the two target sets to eliminate a large proportion of false positives. Proceed to validate the overlapping hits with secondary methods (*see* Note 6).

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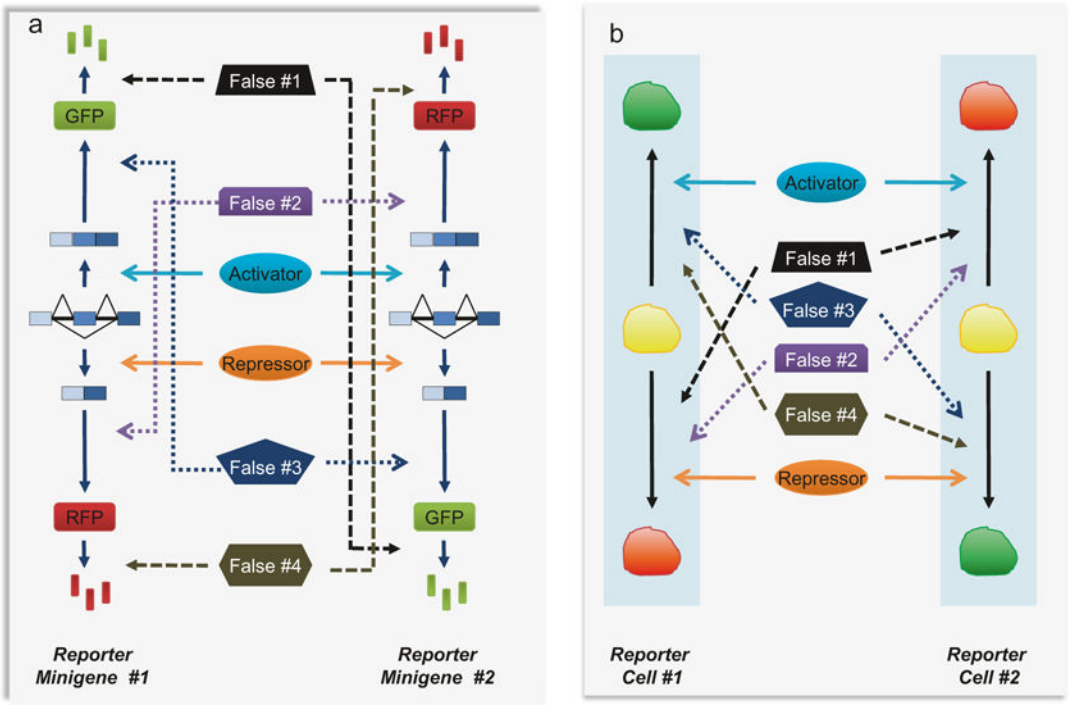

Fig. 1.

Illustration of the IRAS strategy using two dual-fluorescence minigene reporters. (a) Two complementary splicing reporters (#1 and #2) differ in only a few nucleotides. Reporter #1 translates GFP when the alternative exon is included and RFP when the alternative exon is skipped. Reporter #2 translates RFP from the included isoform and GFP from the skipped isoform. The inclusion ratios of the alternative exon in reporter #1 and #2 are therefore expressed by the ratios of GFP/RFP and RFP/GFP respectively. Factors altering these ratios without affecting splicing are false positives in the screens. False positives could differentially promote translation efficiency of GFP (e.g., #3) or RFP (e.g., #2), or alternatively, promote degradation of GFP (e.g., #1) or RFP (e.g., #4). (b) True splicing activators and repressors or false positive hits from screening induce differential phenotypes of shifting the GFP/RFP ratios in the two reporter cells. A true splicing regulator changes GFP/RFP ratios in the two reporter cells in the opposite direction, whereas a false positive more likely affects the GFP/RFP ratios in the same direction

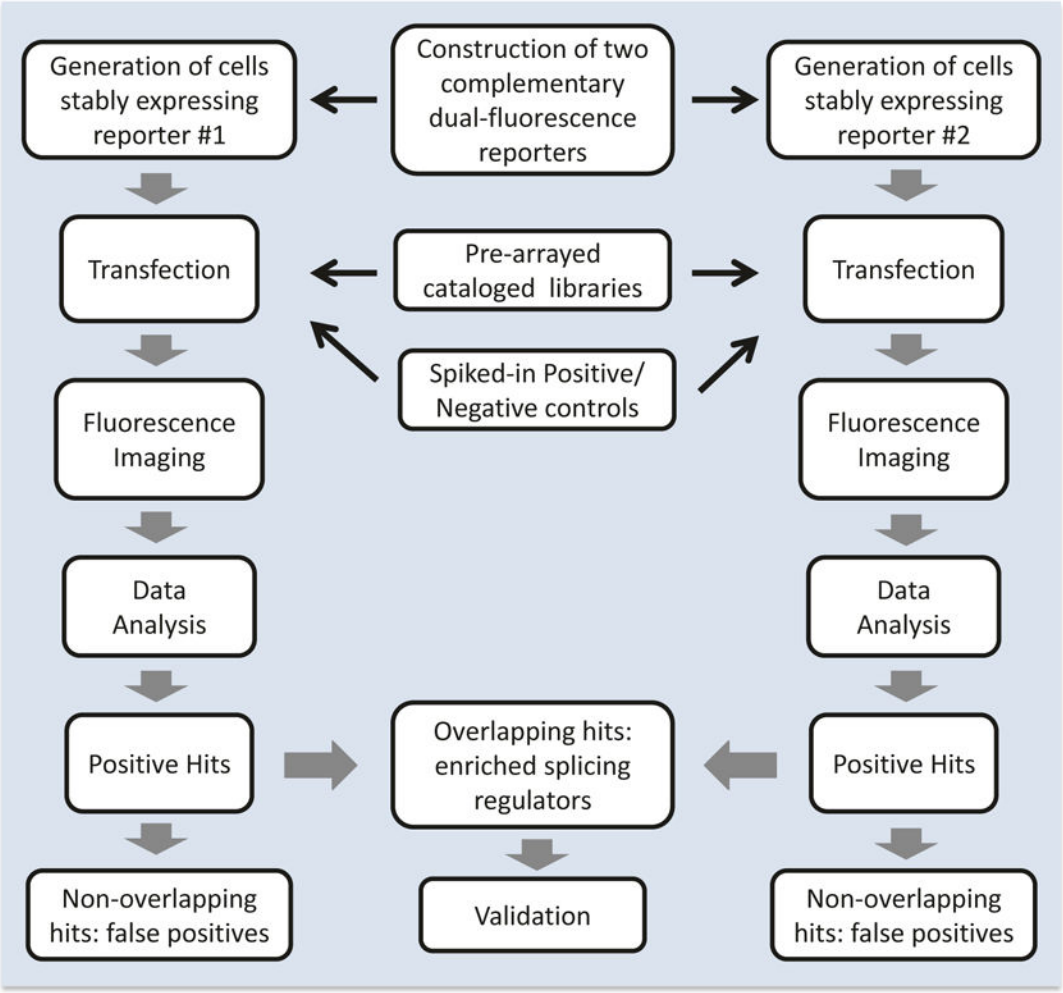

Fig. 2.
Workflow of a fluorescent cell-based high-throughput genetic screen using the IRAS strategy