Receptome Profiling Entered by B. Kang
Janes Lab Protocols 12/06/12

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I. Primer design and validation

1. Identify the gene of interest by its mRNA RefSeq identification (NM_) and paste the sequence into Primer3 (http://frodo.wi.mit.edu/primer3/).
2. Change the following parameters in Primer3:
   - Restrict the size of the amplicon to 150-200 bp — *this size is small enough to amplify easily in a rapid RT-qPCR format but large enough be readily separated from primer dimers by melt-curve analysis.*
   - Restrict the position of the amplicon to within 400 bp on the 3' end of the gene — *this ensures that the Superscript III has reverse transcribed through the region of the gene that will be amplified*
   - *Increase the number of primer pairs returned to 10 — sometimes, the first five primer pairs are not sufficiently different to allow two distinct pairs to be selected*
3. Pick the top-scoring primer pair and BLAST the sequence (fwd_primer NNNN rev_primer) against the appropriate genome. The only perfect matches should be the mRNA and the two genomic sequences from the reference and alternative assemblies.
   - *This BLAST search will identify pseudogenes or other homologous regions that could confound results.*
4. Repeat Step #3 with the next best-scoring primer that is substantially different from the first one.
   - *One primer pair will often work substantially better than the other, so it is cheap enough to order two pairs at the same time.*
5. Make sure that there are no documented SNPs at the 3' end of both forward and reverse primers by manually determining the 3' position of both primers on ApE (or similar software) and checking against dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).
   - *Search by RefSeq ID on NCBI Gene (http://www.ncbi.nlm.nih.gov/gene/), and click on SNP: GeneView under Related information for more reliable access.*
6. Order both pairs of primers at the minimum synthesis scale with standard desalting and dissolve in H_2O at 50 µM.
   - *Use barrier tips to avoid contaminating the primer stocks with PCR amplicons.*
7. Check primer pairs by RT-qPCR (see below) with a blank sample (to check for primer dimers), a no RT sample (to check for pseudogenes or genomic DNA), and a first-strand cDNA sample (to check for amplification).
8. Run the PCR product(s) on 2% agarose gel to determine the product specificity by size.

II. RNA purification and gDNA elimination with RNeasy Plus Mini column chromatography

1. Prepare work area by spraying bench and RNA pipettes with RNAse Away.
2. Cells grown in monolayer: Aspirate the cell culture medium and lyse cells directly in 350 µL (dish diameter < 6cm) or in 600 µL (6–10 cm) buffer RLT Plus.
   Cells grown in suspension: Pellet the cells by centrifuging for 3 min at 300 rcf, aspirate the supernatant, and lyse cells in 350 µL (pelleted cells < 5×10^6 cells) or in 600 µL (5×10^6 – 1×10^7 cells) buffer RLT Plus.
   - *Adherent cells should be 50–80% confluent and suspension cells should be 50–80% of their highest recommended culture density*
   - *It is critical not to lyse more cells or else the gDNA Eliminator spin column will saturate and cause genomic amplification in the no RT controls*
3. Homogenize the sample by pipetting the lysate up and down several times with a P200 micropipette.
4. Transfer the homogenized lysate to a gDNA Eliminator spin column (blue, only in Mini Plus kit) and centrifuge for 30 s at > 8,000 rcf (> 10,000 rpm on a benchtop centrifuge). Discard the column and save the flow-through.
5. Add 1 volume (350 µL or 600 µL) of 70% ethanol to the flow-through and mix well by pipetting.
   - *Do not centrifuge to mix.*

- *White precipitates may appear after adding ethanol depending on the cell line.*

6. Transfer up to 700 µL of the sample to an RNeasy spin column (pink) and centrifuge for 15 s at > 8,000 rcf (> 10,000 rpm on a benchtop centrifuge). Discard the flow-through in guanidinium waste.
   - *It may require two separate spins to process the entire sample*
7. Add 700 µL buffer RW1 to the column and centrifuge for 15 s at > 8,000 rcf (> 10,000 rpm on a benchtop centrifuge). Discard the flow-through in guanidinium waste.
8. Add 500 µL buffer RPE to the column and centrifuge for 15 s at > 8,000 rcf (> 10,000 rpm on a benchtop centrifuge). Discard the flow-through.
   - *Verify that ethanol has been added to RPE buffer before using.*
9. Add 500 µL buffer RPE to the column and centrifuge for **2 min** at > 8,000 rcf (> 10,000 rpm on a benchtop centrifuge). Discard the flow-through.
   - *After this longer spin, the column should be completely dry. If not, spin for an additional minute.*
10. Place the RNeasy spin column in a new 1.5 mL centrifuge tube, add 40 µL of nuclease-free water directly to column resin, and centrifuge for 1 min at > 8,000 rcf (> 10,000 rpm on a benchtop centrifuge) to elute the RNA.
11. Quantify the RNA concentration on a Nanodrop and store at –80°C, or proceed immediately to Step II.
    - *Use RNA pipettes and do not recover sample from Nanodrop to avoid contamination*

III. First-strand cDNA synthesis

1. To 2 µg of purified RNA, add the following:
   2 µL 50 µM oligo$(dT)_{24}$ primer
   2 µL 10 mM dNTPs
   Volume to 26 µL with nuclease-free H_2O
   - *We run the reverse transcription reaction at double the manufacturer's recommended volume, which is sufficient for 400 RT-qPCR experiments (see Janes_RTqPCR.pdf).*
2. Heat denature for 5 min at 65°C on a PCR thermocycler and then place on ice.
3. Prepare the following as a master mix:
   8 µL 5X first-strand buffer
   2 µL 0.1 M DTT
   2 µL RNAsin Plus RNAse inhibitor (Promega #N2615)
   2 µL Superscript III reverse transcriptase (Invitrogen #18080-044)
   14 µL total volume
4. Mix thoroughly by pipetting and then incubate the RT reactions at 50°C for 60 min, followed by heat denaturation at 70°C for 15 min on a PCR thermocycler.
5. Transfer cDNA samples to a fresh microcentrifuge tube and store at –20°C.
   - *Total volume should be 40 µL per sample.*

IV. Plate preparation and storage

1. Prepare primer master mixes (~70 x 3 µL) for corresponding wells in strip tubes.
2. Add 3 µL of primer master mixes into corresponding wells of 96-well plates with a multichannel pipette.
3. Use the plates fresh or seal the plates with plate sealers and store them in –20°C for up to 2 days.
4. (Optional) For indefinite storage of the plates, place up to 6 plates into the lyophilizer vessel, close the vessel, and seal the vessel opening with the parafilm to ensure that the frost does not form.
5. (Optional) Freeze the vessel overnight at –20°C, place the vessel in a Styrofoam box, and put dry ice into the box, making sure that the dry ice covers the vessel.
6. (Optional) Take off the parafilm and hook the vessel up to the lyophilizer and lyophilize for 24 hours at 0.110 mbar.

V. Real-time PCR

1. Mix 10 µL of sample cDNA with 755 µL 2x master mix and 455 µL H_2O (755 µL H_2O for lyophilized plates).
2. Add 150 µL (187 µL for lyophilized plates) of the mixture into each well of a strip tube.

3. Add 12 µL (15 µL for lyophilized plates) of the mixture into each well of a 96-well plate with a multichannel pipette.
   - *Pipet 6 µL (7.5 µL for lyophilized plates) twice if the pipette cannot dispense >10 µL.*
4. Seal the plate completely with optically clear Microseal 'B' film (Biorad #MSB1001)
   - *Pay particularly close attention to the edges and corners of the plate; the reaction will evaporate if not completely sealed and give artifacts*
5. Run the following cycling program on the CFX96 instrument (105°C hotlid)
   - 95°C denaturation, 1.5 min
   - 40 cycles of 95°C denaturation (10 sec), 60°C annealing (10 sec), 72°C elongation (12 sec) with a fluorescence read at the end of the elongation
   - 65°C → 95°C touchup in 0.5°C increments with a fluorescence read after each increment (melt-curve analysis)
6. Compressed .zpcr files can then be imported into the CFX96 reader software for analysis.
7. Run a total of 4 plates per sample.

VI. Data processing

1. Load the .zpcr file onto Bio-Rad CFX Manager and select predesigned plates to create a .pcrd file.
2. Set the baseline threshold of amplification (Quantification) as 25 RFU.
   - *Right click on the amplification curve, select Baseline Threshold, select User Defined, and input 25.*
3. Set the baseline threshold of melt peak curve (Melt Curve) as 15 RFU.
   - *Right click on the melt peak curve, select Show Threshold Values, and move the threshold down until it is at around 15.*
   - *The reduced threshold ensures that all the peaks are captured for future processing.*
4. Export all data (Export -> Export All Data Sheets to Excel).
5. (Recommended) For best compatibility with later processing, convert the exported "Quantitation Ct (or Cq) Results" and "Melt Curve Peak Results" spreadsheets to tab delimited .txt files by opening them on Excel, going to File -> Save As, choosing "Text (tab delimited)" under "Save as type:", and saving.
6. Run receptor_profiling.m on MATLAB to load the GUI.
7. Select appropriate Melt Curve Peak and Quantification Ct (Cq) files and process the files.
8. A new window will appear to confirm that the processing is complete.

VI. Updating the software

1. **Introduction:** The software (`receptor_profiling.m`) may need to be updated by the end-user to make it more robust and to optimize processing of future experiments. Read below to understand the function structure of the software and to update the software.
2. **Function:** This software takes in the Quantitation Ct data and Melt Curve Peak data and outputs Ct values, and melting temperature (Tm) for each gene, positive/negative call, and a note if applicable.
   a. ***Positive/negative call*:** The positive/negative call is made by comparing the detected Tm against a set of validated Tm's for each gene. If the detected Tm matches with one of the validated Tm's, then a positive call is made. If it matches with one of the Tm's found to be of nonspecific amplification, then a negative call is made.
   b. ***"Higher Tm detected…"*:** If the detected Tm is greater than the highest validated Tm and is not one of the Tm's found to be of nonspecific amplification, then a negative call is made with a note "Higher Tm detected. Run on a gel to verify."
   c. ***"Lower Tm detected…"*:** If the detected Tm is 0.5 degrees less than the lowest validated Tm and is not one of the Tm's found to be of nonspecific amplification, then a negative call is made with a note "Lower Tm detected. Run on a gel to verify." This is to ensure that products that are slightly shorter than the expected product are not overlooked, as it is possible for the product to be slightly shorter due to splicing, SNPs, or mutation.
   d. ***"Double-check on a gel"*:** Read the note about the `.lowhigh` vector below.
3. **Structure**: The information regarding the validated melting temperature of each gene's amplified product is stored in the following structure in the `pncall` function:

```
genetm(number).name = 'Gene name';
genetm(number).pos = {set of validated Tm};
genetm(number).neg = {set of Tm found to be of nonspecific
amplification};
genetm(number).lowhigh = {0, 0}
```

Note: The `.lowhigh` vector is designed to strengthen the calls made on amplifications whose Tm is known to have a range of 1 degree or greater. Value 0 is assigned initially. If Tm at either extreme was detected for a gene, then the program will prompt the end user to run the product on a gel to verify specific amplification. If the end user finds that that it was a specific amplification, value 1 should be assigned for the first term if it was for the lower extreme, and for the second term if it was for the higher extreme. If the end user finds that the product of a certain temperature is consistently negative, it is suggested that the temperature be removed from the `.pos` vector.

Example:

```
genetm(23).name = 'EPHB6';
genetm(23).pos = {86.5, 87, 87.5, 88};
genetm(23).neg = {88.5, 89};
genetm(23).lowhigh = {1, 0};
```

In this particular example, if a Tm of 86.5 is detected, a positive call would be made due to the 1 in the "low" position in the `.lowhigh` vector. If a Tm of 88 is detected, however, a positive call would be made but the note "`Double-check on a gel`" would be outputted. If a Tm of 86 is detected, a negative call would be made and the note "`Lower Tm detected…`" would be outputted. However, if a Tm below 86 is detected, a negative call would be made with no note. If a Tm of 88.5 is detected, a negative call would be made as it matches with one of the Tm's in the `.neg` vector. However, if a Tm of 89.5 were to be detected, the note "`Higher Tm detected…`" would be outputted.

4. **Updating protocol**
   a. Open `receptor_profiling.m`.
   b. If the amplicon products with "`Higher Tm detected…`" and "`Lower Tm detected…`" notes are positive after gel electrophoresis, search for the corresponding gene name, and add the detected Tm into the `.pos` vector. If they are negative, add the detected Tm into the `.neg` vector.
   c. If the amplicon products with "`Double-check on a gel`" note are positive after gel electrophoresis, search for the corresponding gene name, determine whether the detected Tm is the lower extreme or higher extreme, and change the corresponding value in `.lowhigh` vector from 0 to 1. As noted above, if they are consistently negative, consider removing the detected Tm from the `.pos` vector and adding it to the `.neg` vector.
   d. (Optional) If the end-user wants to add more genes to the software, simply add more structures to the end of the `pncall` function with increasing structure numbers.

Buffer recipe

- **2× RT-qPCR master mix** Store at –20°C in 985 µL aliquots
  200 µL 10× PCR Buffer II (Applied Biosciences # N8080130 or make from scratch)
  320 µL 25 mM $MgCl_2$
  40 µL 10 mM dNTPs
  30 µL 10 mg/ml BSA
  200 µL 50% glycerol
  195 µL nuclease-free H_2O
  After thawing, add 10 µL 5 U/µL Taq polymerase (Roche #11435094001) and 5 µL 100× SYBR Green (Invitrogen #S7563 diluted 100-fold in DMSO to 100×)
  Store thawed 2× mix at 4°C for 1-2 weeks