

Protocol on U1in = U1inhibitory technique

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last updated July 1, 2003 Sam Gunderson's lab

p702P is vector for cloning

611 oligo is common oligo for PCR

p670U1hind3 is U1 snRNA template plasmid for PCR

Annotation for p702 vector (6487bp)

U1 snRNA gene 25-615 is forward orientation.

U1 promoter is from 25-415

U1 snRNA coding is 416-579.

U1 snRNA 3' termination signal is 585-610.

Bicistronic mRNA

CMV constitutive promoter 932 -1436

GFP coding 1580-2300

IRES 2660-3236

Puromycin Resistance Gene coding 3270-3870

cgcgttacat..... tatataagcag

atggtgagca..... ctgtacaagtaa

ccgcccctctccct..... caccgatgata

atgaccgagta... cccggtgcctga

Col E1 origin of replication 4730-5310

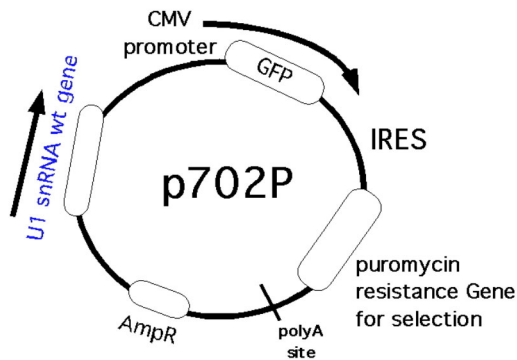
Amp Resistance (reverse strand) 6351-5491

Promoter for Amp Gene 6420-6360

gtttttccatag..... atctcaagaagatc

atgagtattcaacat ... ttaagcattggtaa

tcaaatatgtatc.... caataaccctg



Other Notes

Inhibition by transient transfection is more "difficult" than with Stables.

The reason is the 5' end mutated U1 snRNA takes a relatively long time to assemble into a mature 5' end mutated U1 snRNP. The assembly/maturation pathway has the following steps.

Transcription of pre-U1 snRNA, export to cytoplasm, trimming off extra nucleotides, addition of trimethyl cap and modification of nucleotides, assembly of ten U1 snRNP proteins and reimport into nucleus. Only when the matured 5' end mutated U1 snRNP is reimported into the nucleus will inhibition occur. The total time of this maturation is not precisely known but the best estimates indicate 16 hours.

Thus if you co-transfect a reporter plasmid with the 5' end mutated U1 snRNA plasmid the reporter will have a significant "head start" unless you have an inducible reporter (eg. Tet promoter). Thus the inhibition is not as strong as it could be.

A brief schematic for how to generate Stables.

Note that stables can be generated rather quickly with Puromycin as compared to Neomycin or Hygromycin. Preparation: first linearize your 702P/U1 plasmid DNA with Ssp1 or Pvu1. This improves the odds that the integration will not disrupt the U1 snRNA gene or Puromycin gene. Ssp1 or Pvu1 disrupt expression of the Amp gene which does not matter for mammalian cells.

Day 1 split cells

Day 2 Txfect linearized plasmid (Ssp1 or Pvu1) by whatever method is good for your cells.

Day 4 remove Txfaction reagents and plasmid and put cells into growth media

Day 5 add in puromycin to 0.5 $\mu\text{g}/\text{ml}$ (dont add fresh media)

Day 7 change media and add fresh media+ puromycin.

Change the media every 2nd or 3rd day until 95% of cells are dead (usually by day 12).

Once cells are nearly dead then change media every 5-7 days.

By day 20 you should have survivor colonies (50-500 cells per colony).

Pick colonies and transfer to 48 well or 24 well dishes with media+puromycin.

When you have enough cells then screen by RTPCR for housekeeping gene and for target gene.

I recommend screening 30 clones so that you have good odds that one clone is down by 10 fold for target gene.

If your gene is essential then you can replace the GFP coding region of 702P with the coding region of your gene (with epitope tag) under a regulatable promoter (eg. Tet). This allows you to add back your gene while also inhibiting the endogenous gene. Once the stables are made then you can turn off your gene.

Rules for choosing the target sequence on your target gene.

1. Recommended or Required Sequence Features

1A) Length of target site is 10 nts.

1B) Keep the 10nt target on the mRNA balanced in its G-C and A-U content. This means no less than 3 GC's and no more than 7 GC's.

1C) Sequence must be in terminal exon and preferably close (10-200nts) to the polyA signal which for 70% of vertebrate genes is an AAUAAA or AUUAAA sequence about 20nts upstream of the polyA tail addition site. If you are unsure of the position of the last 3' splice site or the polyA signal then choose the 10nt site anywhere in the 3' UTR.

2. Things to avoid.

2A) Avoid regions of strong RNA secondary structure (eg. stem loops). We recommend running an RNA folding program on the entire 3' terminal exon or at least on the 200nts flanking the 10nt target site. An easy-to-use web site where you simply paste in your DNA sequence can be found at http://www.genebee.msu.su/services/rna2_reduced.html.

2B) Avoid repetitive sequences within the 10nt target site.

2C) Avoid runs of A's C's G's U's and palindromes.

2D) Avoid sequences too similar to the natural consensus 5'ss (CAG/GTGAGT). Especially avoid sites having the near universally conserved "GT" at positions 4-5.

2E) **Avoid highly repetitive sequences.** Examples would include LINE elements and ALU repeats that are especially prevalent in the human genome (up to one million copies) but similar repetitive sequences are found in most vertebrate genomes. We recommend taking the entire 3' terminal exon (or at least 200nts flanking the 10nt target site) and doing a BLAST search to see if it is highly repetitive. For example the p53 gene's 3'UTR has a 700nt LINE element that has 1000's of repeats in the human genome.

3. One option that we recommend is to make 3 different U1 snRNA plasmids

each targeting a different part of the terminal exon. That way if one of the U1 snRNA plasmids fails to inhibit you still have two U1 plasmids that inhibit. That way these two U1 snRNA plasmids can also be co-expressed for enhanced synergistic inhibition if needed.