

Example of overgo construction:

8) NF1

```
BASE COUNT      269 a      165 c      190 g      233 t
301 atttatgaac  cttttgaatg  actgcagtga  agttgaagat  gaaagtgcgc  aaacagggtgg
361 caggaaacgt  ggcattgtctc  ggaggctggc  atcactgagg  cactgtacgg  tccttgcaat
421 gtcaaaactta  ctcaatgcc  acgtagacag  tgggtctcatg  cactccatag  gtgagatcaa
481 atgaaagttt  catatagaaa  tacacaacct  agagaactgg  catgtaagag  aagcaaaaat
541 tacttcagca  aggccatggt  agtaaatttn  catctgtttg  tccacattag  gcttaggtta
601 ccacaaggat  ctccagacaa  gagctacatt  tatggaagt  ctgacaaaaa  tccttcaaca
661 aggcacagaa  tttgacacac  ttgcagaaac  agtattggct  gatcggtttg  agagattggg
721 ggaactggtc  acaatgatgg  gtgatcaagg  agaactccct  atagcgatgg  ctctggccaa
781 tgtggttcct  tgttctcagt  gggtaagtga  ttagagtaag  cggggaagan  aagtccctgg
841 cacatagcaa  atccttcaga
```

bold = complete 40mer overgo sequence

NF1F1: gcaggaaacgtggcat**tgtctcgg**

NF1R1: cagtgatgccagcct**ccgagaca**

```
Query:      1 CAGGAAACGTGGCATGTCTCGGAGGCTGGCATCACT 36
            |||
M.mus:    3346 CAGGAAACGTGGCATGTCTCGGAGGCTGGCATCCCT 3381
```

97%

- 4) Assign overgo primer sequences with ID# and alias names.
- 5) Send primer sequences via email to research genetics @ \$.50/base for production.

orders@resgen.com

Solutions

Hybridization solution I (7% SDS)

1mM EDTA
7% SDS (use 99.9% pure SDS)
0.5M Sodium phosphate

1M Sodium phosphate, pH 7.2:

268g Na₂HPO₄·7H₂O in 1700ml H₂O
add 8 ml 85% H₃PO₄ and make to 2000ml

0.5M EDTA, pH 8.0:

93g EDTA (disodium dihydrate) in 400ml H₂O
pH to 8.0 with 6M NaOH and make to 500 ml

To make 4000ml: (use autoclaved ddH₂O)

1. To 2000ml 1M sodium phosphate, add 1200ml H₂O, 8ml 0.5M EDTA and 280g SDS.
2. Heat and stir until the SDS is dissolved (1 hr or so).
3. Bring volume to 4000ml.
4. Warm to 60C before using.

Washing Buffer B: 1 % SDS, 40mM NaPO₄

4x: 48ml EDTA
240g SDS
960ml 1M NaHPO₄, pH7.2
to 6L with ddH₂O

Washing solution 2: 1.5x SSC, 0.1% SDS

1125ml 20x SSC
150ml 10% SDS
to 15L with ddH₂O

Washing solution 3: 0.5x SSC, 0.1% SDS

375ml 20x SSC
150ml 10% SDS
to 15L with ddH₂O

20x SSC:

701.2g NaCl
352g NaCitrate (Na₃.2H₂O)
to 4L with ddH₂O
pH to 7.0 with 6M HCl

10% SDS: 100g SDS/1L ddH₂O

Stripping buffer: 0.1x SSC, 0.1% SDS

2% BSA: 200mg BSA/10ml ddH₂O

10ml 20x SSC
20ml 10% SDS
to 2L with ddH₂O

Overgo labeling buffer: OLB{-A,-C,-N6}

Solution O:	1.25mM Tris-HCl, pH 8.0 125mM MgCl ₂	15.1g Tris-base 2.54g MgCl ₂ ·6H ₂ O to 100ml H ₂ O with ddH ₂ O pH to 8.0 with 6M HCl
Solution A:	1ml solution O 18ul 2-mercaptoethanol 5ul 0.1M dTTP 5ul 0.1M dGTP	
Solution B:	2M HEPES-NaOH, pH 6.6	2.6g HEPES to 5ml ddH ₂ O pH to 6.6 with ~2 drops 6M NaOH
Solution C:	3mM Tris-HCl, pH 7.4 0.2mM EDTA	36mg Tris-base 7mg EDTA to 100 ml ddH ₂ O, pH to 7.4 with 1M HCl

Prepare OLB: A:B:C, 1:2.5:1.5

Solution A	1ml
Solution B	2.5ml
Solution C	1.5ml

Aliquot and store at -20C.

Overgo Labeling Reaction: 10 ul reaction (See below for 50 ul, 6x6x6 array reactions)

- 1) Combine 0.5ul of complimentary 20uM oligos (0.5 + 0.5) with 4.5 ul ddH₂O.
{ 10 pmol each oligo per reaction }
- 2) Heat solution of mixed oligos at 80C, 5 min., 37C, 10 min. Store on ice.
- 3) Add to the oligo mix:

BSA (2%):	0.5ul	
OLB{-A,-C,-N6}	2.0ul	
32P-dATP*	0.5ul	*3000 Ci/mmol, 10 mCi/ml
32P-dCTP*	0.5ul	
Klenow fragment	1 ul (2U/ul)	

- 4) Incubate at room temperature for 1 hour.
- 5) Remove unincorporated nucleotides with Sephadex G50 columns.

Hybridization of Overgos to BAC filters

- 1) Filters are stacked 4-6 high, interleaved with nylon spacers of equal size, in a plastic tray soaked with hyb solution. Press out air bubbles and loosely roll the filters. Insert the rolled filters into the hyb bottle the same way for all bottles. Remove air bubbles from the filters using a 25ml glass pipet, making sure filters are flat. Add 25ml of warmed hyb solution to each bottle. Make sure that all filters are rolled in the same direction and that the bottle is correctly placed in the oven to keep them rolled. The rotation speed is set to 6 speed.
- 2) Filters are prehyb'd for 4 hours the first time used and 1-2 hours thereafter at 60C. Replace hyb solution before adding probes if filter is used for the first time.
- 3) After filter prehybridization, denature the labeled probes by boiling for 10 min. Then immediately place probes on slushy ice and add to appropriate hyb bottle. Probes are allowed to hybridize with filters overnight; however, 2 day hybridizations give somewhat stronger signals, especially with older filters.

Washing: Preheat all wash solutions to 60C

- 1) Hyb solution is removed and the bottle filled with 100ml 1x washing buffer B. The bottle is returned to the oven at 60 C and rotated for 30 minutes, speed 8. All hyb solutions and first round washes are radioactive and disposed of in liquid RAD waste container.
- 2) Filters are then washed as follows in hyb oven, speed 8: (80ml wash)

2x Washing solution 2 at 60 C for 20min
- 3) Remove filters from hyb bottles and wash in shaking water bath for 5 minutes at 60 C with 2.5 L wash solution 3, shaking slowly. Remove nylon spacers before this final wash. **DO NOT OVERWASH.**
- 4) Soak filters in hyb solution.

PhosphoImaging:

- 1) Place filters in plastic bags and remove all air bubbles. Seal and check for leaks. Wipe outside of bag with wet kimwipe to remove any hyb solution.
- 2) Place filters in a phosphoimage cassette for overnight exposure. Scan image into Typhoon imager using: template = filter, pixel = 100um. (13 minutes to scan image)
- 3) After image capture, filters are stored at -20C in the sealed plastic bag.

Positive Identification:

Positive clones are identified by the presence of duplicate signals using ArrayVision software, and the corresponding clone addresses are exported to the IDProbes program for deconvolution of the clones that are positive at three intersection points. Positive clones are then are picked, cultured, and prepped for DNA. See ArrayVision protocol.

PCR verification and/or fingerprinting are then carried out to verify clone/loci assignment.

Stripping filters: (not routinely done)

Filters are stripped of previously bound overgos by:

Place filters in 1.5 liters of stripping buffer for 30 minutes at 70 C at moderate speed. Check for counts with survey meter to verify efficacy of stripping procedure. Repeat for additional 10 minutes if necessary. DO NOT OVERSTRIP. Keep temperatures at 70 C and do not reduce salt concentration of stripping buffer. Overstripping will remove BAC DNA and reduce life of filters.

1. Hybridization based screening of RPCI-11 Segment 1 High-Density filters using overlapping oligonucleotide probes (overgos) in a 6 x 6 x 6 array.

Overgo based screening of RPCI-11 high density filters is accomplished by hybridizing pools of 36 overgos in a 6 x 6 x 6 array, utilizing 216 probes. Each round of hybridization with 36 pooled overgos provides within the same set of experiments a third confirming intersection point, thereby greatly reducing the number of false positives. Labeling enough overgos for all three rounds of hybridization eliminates probe variability between reactions.

Overgo labeling reaction: 50 ul reaction

- a. Combine 2.5ul of complimentary 20uM oligos (2.5 ul forward primer + 2.5ul reverse primer) with 22.5ul ddH2O. Array the 216 oligo pairs into a 6 x 6 x 6 panel. See example below.
- b. Heat solution of paired oligos at 80C, 5 min., 37C, 10 min. Store on ice.
- c. Add 22.5 ul of Klenow reaction mix to the oligos: (make enough for 250 reactions)

<u>Klenow reaction mix: 1 RXN</u>		<u>250 RXN</u>	
BSA (2%)	2.5ul	625 ul	
OLB{-A,-C,-N6}	10 ul	2500 ul	
32P-dATP*	2.5ul	625 ul	*3000 Ci/mmol, 10mCi/ml
32P-dCTP*	2.5ul	625 ul	
Klenow fragment	5.0ul (2U/ul)	1250 ul (2500 U)	

- d. Incubate at room temperature for 1 hour.

e. **ANCHOR SPOTS:**

When anchor spots are available on the hybridization filter, an additional “anchor-spot” overgo can be included in each hybridization bottle. The anchor clones are spotted in diagonal pairs in each corner of each filter panel, resulting in 24 pairs of positive signal. The positive anchor clones provide anchors for the proper, automated alignment of the ArrayVision filter templates. This reduces the time spent aligning the templates on the various filter images encountered and allows for rapid scoring of filters with minimal background.

A typical 6x6x6 array of overgos requires 18 separate hybridizations; and therefore, requires 10ul x 18 = 180 ul of labeled overgo. The overgo oligos used are **PDJ11592 and PDJ11593**. These oligos contain sequences homologous to the C.briggsae clone RPCI-94 1a1T, which is the source of the anchor clone.

Anchor Spot Overgo Labeling Reaction: 200 ul reaction

- 1) Combine 10ul of complimentary 20uM oligos (10 + 10) with 90 ul ddH₂O.
- 2) Heat solution of mixed oligos at 80C, 5 min., 37C, 10 min. Store on ice.
- 3) Add to the oligo mix:

BSA (2%):	10ul	
OLB{-A,-C,-N6}	40ul	
32P-dATP*	10ul	*3000 Ci/mmol, 10 mCi/ml
32P-dCTP*	10ul	
Klenow fragment	20 ul (2U/ul)	

- 4) Incubate at room temperature for 1 hour.
- 5) Remove unincorporated nucleotides with Sephadex G50 columns as described below.

f. **Remove unincorporated radionucleotides with Sephadex G50 columns**

- 1) To check overgo probe incorporation of radionucleotides, aliquot 1 ul of unpurified overgo mix to 2 ml scintillation fluid. Repeat for all 216 probes.
- 2) To 96-well PCR plates, poke holes in the bottom with an 18-gauge needle and then add glass beads followed by 200 ul of sephadex slurry. Centrifuging at 1000 rpm x 10 sec, collecting eluate in another 96-well PCR plate. Check columns for uniform resin height and thorough removal of eluate. Replace collection tubes with new collection tubes.
- 3) Immediately, add the overgo mix, spin at 2000 rpm for 2 min, collecting all purified probe in 96-well collection tubes.
- 4) Aliquot 1 ul of purified overgo probe to 2 ml scintillation fluid. Repeat for all 216 probes.
- 5) Count all vials for 20 seconds in scintillation counter.
- 6) Calculate percent incorporation

g. Combine purified overgos into hybridization pools: (example below)

Overgo Probe Arrays:

	COLUMN	1	2	3	4	5	6
ROW	POOL	99	100	101	102	103	104
1	93	314	315	316	317	318	319
2	94	320	321	322	323	324	325
3	95	326	327	328	329	330	331
4	96	332	333	334	335	336	337
5	97	338	339	340	341	342	343
6	98	344	345	346	347	348	349

PLATE 1

	COLUMN	1	2	3	4	5	6
ROW	POOL	111	112	113	114	115	116
1	105	350	351	352	353	354	355
2	106	356	357	358	359	360	361
3	107	362	363	364	365	366	367
4	108	368	369	370	371	372	373
5	109	374	375	376	377	378	379
6	110	380	381	382	383	384	385

PLATE 2

	COLUMN	1	2	3	4	5	6
ROW	POOL	123	124	125	126	127	128
1	117	386	387	388	389	390	391
2	118	392	393	394	395	396	397
3	119	398	399	400	401	402	403
4	120	404	405	406	407	408	409
5	121	410	411	412	413	414	415
6	122	416	417	418	419	420	421

PLATE 3

	COLUMN	1	2	3	4	5	6
ROW	POOL	135	136	137	138	139	140
1	129	422	423	424	425	426	427
2	130	428	429	430	431	432	433
3	131	434	435	436	437	438	439
4	132	440	441	442	443	444	445
5	133	446	447	448	449	450	451
6	134	452	453	454	455	456	457

PLATE 4

	COLUMN	1	2	3	4	5	6
ROW	POOL	147	148	149	150	151	152
1	141	458	459	460	461	462	463
2	142	464	465	466	467	468	469
3	143	470	471	472	473	474	475
4	144	476	477	478	479	480	481
5	145	482	483	484	485	486	487
6	146	488	489	490	491	492	493

PLATE 5

	COLUMN	1	2	3	4	5	6
ROW	POOL	159	160	161	162	163	164
1	153	494	495	496	497	498	499
2	154	500	501	502	503	504	505
3	155	506	507	508	509	510	511
4	156	512	513	514	515	516	517
5	157	518	519	520	521	522	523
6	158	524	525	526	527	528	529

PLATE 6

Overgo Hybridization Pools:

HYB #1: PLATE POOLS

BTL #:	POOL:		FILTER BARCODE:
1	93-98	1036	6136(1F), 6164(2F), 6188(3F), 6212(4F), 6236(5F), 6260(6F)
2	105-110	1038	6138(1H), 6166(2H), 6190(3H), 6214(4H), 6238(5H), 6262(6H)
3	117-122	1039	6139(1I), 6167(2I), 6191(3I), 6215(4I), 6239(5I), 6263(6I)
4	129-134	1040	6140(1J), 6168(2J), 6192(3J), 6216(4J), 6240(5J), 6264(6J)
5	141-146	1041	6141(1K), 6169(2K), 6193(3K), 6217(4K), 6241(5K), 6269(6C)
6	153-158	1044	6144(1B), 6172(2B), 6196(3B), 6220(4B), 6244(5B), 6268(6B)

HYB #2: ROW POOLS

BTL #:	POOL:		FILTER BARCODE:
1	93,105,117,129,141,153,	1046	6146(1D), 6174(2D), 6198(3D), 6222(4D), 6246(5D), 6270(6D)
2	94,106,118,130,142,154,	1047	6147(1E), 6175(2E), 6199(3E), 6221(4C), 6247(5E), 6271(6E)
3	95,107,119,131,143,155,	1048	6148(1F), 6176(2F), 6200(3F), 6224(4F), 6248(5F), 6272(6F)
4	96,108,120,132,144,156,	1049	6149(1G), 6177(2G), 6201(3G), 6225(4G), 6249(5G), 6273(6G)
5	97,109,121,133,145,157,	1109	6576(1D), 6588(2D), 6600(3D), 6612(4D), 6624(5D), 6636(6D)
6	98,110,122,134,146,158,	1110	6577(1E), 6589(2E), 6601(3E), 6613(4E), 6625(5E), 6637(6E)

HYB #3: COLUMN POOLS

BTL #:	POOL:		FILTER BARCODE:
1	99,111,123,135,147,159,	1373	7957(1A), 7981(2A), 8005(3A), 8029(4A), 8069(5A), 8097(6A)
2	100,112,124,136,148,160,	1386	7969(1A), 7993(2A), 8017(3A), 8041(4A), 8081(5A), 8109(6A)
3	101,113,125,137,149,161,	1387	7970(1B), 7994(2B), 8018(3B), 8042(4B), 8082(5B), 8110(6B)
4	102,114,126,138,150,162,	1388	7971(1C), 7995(2C), 8019(3C), 8043(4C), 8083(5C), 8111(6C)
5	103,115,127,139,151,163,	1389	7972(1D), 7996(2D), 8020(3D), 8044(4D), 8084(5D), 8112(6D)
6	104,116,128,140,152,164,	1390	7973(1E), 7997(2E), 8021(3E), 8045(4E), 8085(5E), 8113(6E)

Hybridizations are completed within a week of radiolabeling the overgos to insure high specific probe activity.

Positive clones are scored by ArrayVision and exported into an Identify Probe program. The program assigns clones to their respective probes and assimilates the data in a user-friendly format. Statistical analysis of the data provides useful measures of filter:filter variability and overall quality control.

Positive clone DNA is re-arrayed into 96-well plates by ascending clone number and stored as glycerol cell cultures and DNA stocks at -80C.