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Analyzing Myc in Cell Transformation and Evolution

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Abstract

The *myc* oncogene was originally identified as a transduced allele (*v-myc*) in the genome of a highly oncogenic avian retrovirus. The protein product (Myc) of the cellular *c-myc* protooncogene represents the key component of a transcription factor network controlling the expression of a large fraction of all human genes. Myc regulates fundamental cellular processes like growth control, metabolism, proliferation, differentiation, and apoptosis. Mutational deregulation of *c-myc* leading to increased levels of the Myc protein is a frequent event in the etiology of human cancers. In this chapter, we describe cell systems and experimental strategies to monitor and quantify the oncogenic potential of *myc* alleles, and to isolate and characterize transcriptional targets of Myc that are relevant for the cell transformation process. We also describe experimental procedures to study the evolutionary origin of *myc* and to analyze structure and function of the ancestral *myc* protooncogenes.

Keywords

Oncogene; Myc; Max; Transcription factor; Cell transformation; Cancer; Evolution; Protein-DNA interactions; Target gene

1 Introduction

The *myc* oncogene was originally identified as the transforming principle (*v-myc*) in the genome of avian acute leukemia virus MC29 encoding a single hybrid protein composed of partial structural (Gag) and Myc sequences (1, 2). The highly oncogenic *v-myc* allele is derived from the chicken *c-myc* protooncogene by retroviral transduction (3, 4). Subsequently, homologs of *c-myc* were identified in all vertebrate genomes. The protein product (Myc) of *c-myc* represents the key component of a transcriptional regulator network controlling the expression of a large fraction of all human genes (5, 6). Myc is involved in the regulation of fundamental cellular processes like growth control, metabolism, proliferation, differentiation, and apoptosis. Deregulation of *c-myc* leading to increased levels of the Myc protein is a frequent mutational event in human tumorigenesis, occurring in about 30% of all human cancers (4, 7). Myc is a bHLH-Zip protein containing protein dimerization domains (helix-loop-helix and leucine zipper) and an adjacent DNA contact surface (basic region). Myc forms heterodimers with the bHLH-Zip protein Max, binds to specific DNA sequence elements (E-boxes) with the preferred consensus sequence 5'-

CACGTG-3', and is part of a transcription factor network including additional bHLH-Zip proteins (5, 6, 8). Myc-Max heterodimers are involved in transcriptional activation of distinct target genes, but Myc has also been implicated in transcriptional repression. Several genes activated by Myc are related to processes of cell growth and metabolism, including protein synthesis, ribosomal biogenesis, glycolysis, mitochondrial function, and cell cycle progression (6, 9). Most of the genes repressed by Myc are involved in cell cycle arrest, cell adhesion, and cell-to-cell communication (6). Invertebrate orthologs of Myc and Max proteins have been identified in the triploblastic bilaterian organism *Drosophila melanogaster* (10). *Drosophila* dMyc and dMax bind to a large number of E-boxes in the *Drosophila* genome and regulate the expression of many genes including key regulators of cell growth, cell size, and ribosome biogenesis (11). Recently, ancestral forms of *myc* and *max* genes have been identified in the early diploblastic cnidarian *Hydra*, the most primitive metazoan organism employed so far for the structural, functional, and evolutionary analysis of these genes (12). The principal design and the basic biochemical properties of the *Hydra* Myc and Max proteins are very similar to those of their vertebrate derivatives, suggesting that the principal functions of the Myc master regulator arose very early in metazoan evolution, at least 600 million years ago.

Here we describe the essential methods to monitor and quantify the oncogenic potential of *myc* alleles in an avian fibroblast cell transformation system and to use that system to isolate and characterize target genes that are transcriptionally regulated by the Myc protein. Furthermore, we refer to methods to detect and analyze *myc* genes and their expression in evolutionary simple metazoan organisms.

2 Materials

All buffers, media, and solutions used for the procedures described in the Methods section (see below) are listed in alphabetical order.

1. 30% (w/v) Acrylamide / 0.8% (w/v) bisacrylamide. Dissolve 30 g acrylamide and 0.8 g bisacrylamide in H₂O to 100 ml; filtrate (0.45 µm); store light-protected at 4°C.
2. 1% (w/v) Agarose / formaldehyde gel: 1% (w/v) agarose, 6.57% (v/v) formaldehyde, 1x RNA gel running buffer. Suspend 2 g agarose in 144 ml H₂O and boil in a microwave oven; cool to 65°C; add 20 ml 10x RNA gel running buffer and 36 ml formaldehyde solution (36.5%) warmed to 65°C; mix and pour the gel (200 ml) in a chemical hood.
3. 10x Annealing buffer: 100 mM Tris HCl pH 8.0, 50 mM MgCl₂, 650 mM KCl, 5 mM EDTA, 10 mM DTT. Mix 180 µl H₂O, 100 µl 1 M Tris HCl pH 8.0, 50 µl 1 M MgCl₂, 650 µl 1 M KCl, 10 µl 0.5 M EDTA, and 10 µl 1 M DTT solution; store at -20°C.
4. 1 mg/ml Aprotinin. Dissolve 5 mg aprotinin from bovine lung in 10 mM HEPES pH 7.9 to 5 ml; store in 1-ml aliquots at -20°C.

5. 10% (w/v) APS. Dissolve 1 g APS (ammonium peroxydisulfate) in H₂O to 10 ml; store in 1-ml aliquots at -20°C.
6. Avian cell culture medium: 1x Ham's F10 with glutamine, 0.295% (w/v) TPB, 5% (v/v) calf serum, 2% (v/v) chicken serum, 0.188% (w/v) NaHCO₃, 0.5% (v/v) DMSO, 1x antibiotic-antimycotic. Mix 345 ml H₂O, 50 ml 10x Ham's F10 with glutamine, 50 ml 2.95% (w/v) TPB, 25 ml calf serum, 10 ml chicken serum (heat-inactivated at 56°C for 30 min), 12.5 ml 7.5% (w/v) NaHCO₃, 2.5 ml DMSO (dimethyl sulfoxide), and 5 ml 100x antibiotic-antimycotic; store at 4°C.
7. 1.8% (w/v) Bacto agar. Suspend 1.8 g bacto agar in 100 ml H₂O; autoclave; store at 4°C.
8. Boiling buffer: 10 mM sodium phosphate pH 7.2, 0.5% (w/v) SDS, 2 µg/ml aprotinin. Mix 9.65 ml H₂O, 100 µl 1 M sodium phosphate pH 7.2, and 250 µl 20% (w/v) SDS; store at RT; prior to use add 1/500 vol 1 mg/ml aprotinin.
9. Buffered F10 medium: 1x Ham's F10 with glutamine, 0.188% (w/v) NaHCO₃, 25 mM HEPES pH 7.3. Mix 425 ml H₂O, 50 ml 10x Ham's F10 with glutamine, 12.5 ml 7.5% (w/v) NaHCO₃, and 12.5 ml 1 M HEPES pH 7.3; store at 4°C.
10. 2.5 M CaCl₂. Dissolve 18.38 g CaCl₂ (calcium chloride) x 2 H₂O in H₂O to 50 ml; filter sterilize (0.22 µm); store at 4°C.
11. 1x Cell culture lysis reagent: 25 mM Tris phosphate pH 7.8, 2 mM DTT, 2 mM DCTA (trans-1,2-diaminocyclohexane-N,N',N'-tetraacetic acid), 10% (v/v) glycerol, 1% (v/v) Triton X-100 [4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol]. Supplied by the Luciferase assay system E1500 (Promega); store at -20°C.
12. ChIP-dilution buffer: 0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl pH 8.0, 167 mM NaCl, 1 mM PMSF, 2 µg/µl aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A. Mix 167.4 ml H₂O, 100 µl 20% (w/v) SDS, 22 ml 10% (v/v) Triton X-100, 480 µl 0.5 M EDTA, 3.34 ml 1 M Tris HCl pH 8.0, and 6.68 ml 5 M NaCl; filter sterilize (0.22 µm); store at 4°C; prior to use add 1/100 vol PMSF, 1/500 vol aprotinin, 1/1000 vol leupeptin, 1/1000 vol pepstatin A.
13. ChIP-protein A-bead slurry: 0.005% (w/v) SDS, 0.55% (v/v) Triton X-100, 0.6 mM EDTA, 8.35 mM Tris HCl pH 8.0, 83.5 mM NaCl, 0.2 µg/µl salmon sperm DNA, 0.5 µg/µl BSA, 50% (v/v) protein A sepharose CL-4B. Suspend 200 mg protein A sepharose CL-4B beads in 10 ml H₂O and incubate on ice for 30 min; wash 3 x with each 10 ml H₂O (during the washing the beads should swell on ice for 30 min), then 1 x with 2 ml of ChIP-dilution buffer; to the swollen pellet (~ 1 ml) add 1 ml of ChIP-dilution buffer, 40 µl of 10 mg/ml sonicated salmon sperm DNA, and 100 µl of 10 mg/ml BSA; store at 4°C.
14. ChIP-elution buffer: 1% (w/v) SDS, 0.1 M NaHCO₃. Mix 42.5 ml H₂O, 2.5 ml 20% (w/v) SDS, and 5 ml 1 M NaHCO₃; filter sterilize (0.22 µm); store at RT

15. ChIP-low salt washing buffer: 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris HCl pH 8.0, 150 mM NaCl. Mix 168.2 ml H₂O, 1 ml 20% (w/v) SDS, 20 ml 10% (v/v) Triton X-100, 800 µl 0.5 M EDTA, 4 ml 1 M Tris HCl pH 8.0, and 6 ml 5 M NaCl; filter sterilize (0.22 µm); store at 4°C.
16. ChIP-high salt washing buffer: 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris HCl pH 8.0, 500 mM NaCl. Mix 154.2 ml H₂O, 1 ml 20% (w/v) SDS, 20 ml 10% (v/v) Triton X-100, 800 µl 0.5 M EDTA, 4 ml Tris HCl pH 8.0, and 20 ml 5 M NaCl; filter sterilize (0.22 µm); store at 4°C.
17. ChIP-LiCl washing buffer: 0.25 M LiCl, 1% (w/v) Igepal-CA630, 1% (w/v) sodium deoxycholate, 1 mM EDTA, 10 mM Tris HCl pH 8.0. Mix 147.6 ml H₂O, 10 ml 5 M LiCl, 20 ml 10% (v/v) Igepal-CA630 (octylphenyl-polyethylene glycol), 20 ml 10% (w/v) sodium deoxycholate, 400 µl 0.5 M EDTA, and 2 ml 1 M Tris HCl pH 8.0; filter sterilize (0.22 µm); store at 4°C.
18. Cloning bottom agarose: 1x Ham's F10 with glutamine, 0.295% (w/v) TPB, 3.5% (v/v) calf serum, 1.25% (v/v) chicken serum, 0.188% (w/v) NaHCO₃, 1.25% (v/v) DMSO, 1x glutamine, 1x antibiotic-antimycotic, 0.625% (w/v) sea plaque agarose. Heat 1 vol 1.5% (w/v) sea plaque agarose in a microwave oven; cool to 45°C in a water bath; mix with 45°C warm 1.4 vol DC3 medium; keep at 45°C in a water bath.
19. Cloning medium: 1.25x Ham's F10 with glutamine, 0.369% (w/v) TPB, 12.5% (v/v) calf serum, 5.75% (v/v) chicken serum, 0.25% (v/v) DMSO, 1.875x vitamins solution, 1.875x folic acid solution, 1.25x glutamine, 1.25x antibiotic-antimycotic. Mix 201 ml H₂O, 50 ml 10x Ham's F10 with glutamine, 50 ml 2.95% (w/v) TPB, 50 ml calf serum, 23 ml chicken serum (heat-inactivated at 56°C for 30 min), 1 ml DMSO, 7.5 ml 100x vitamins solution, 7.5 ml 100x folic acid solution, 5 ml 100x L-glutamine (200 mM), and 5 ml 100x antibiotic-antimycotic; store at 4°C.
20. Cloning top agarose: 1x Ham's F10 with glutamine, 0.295% (w/v) TPB, 10% (v/v) calf serum, 4.6% (v/v) chicken serum, 0.2% (v/v) DMSO, 1.5x vitamins solution, 1.5x folic acid solution, 1x glutamine, 1x antibiotic-antimycotic, 0.3% (w/v) sea plaque agarose. Heat 1 vol 1.5% (w/v) sea plaque agarose in a microwave oven; cool to 45°C in a water bath; mix with 45°C warm 4 vol cloning medium; keep at 45°C in a water bath.
21. CsCl buffer ($d = 1.71 \text{ g/cm}^3$): 5.7 M CsCl, 25 mM sodium acetate pH 6.0. Dissolve 95.97 g CsCl (cesium chloride) in H₂O to 99.17 ml; add 0.83 ml 3 M sodium acetate pH 6.0; filter sterilize (0.22 µm); autoclave; store at RT; check the density (d) by determination of the refractory index (n) using a refractometer : $d = (10.8601 \times n) - 13.4974$.
22. DC3 medium: 1.714x Ham's F10 with glutamine, 0.506% (w/v) TPB, 6.02% (v/v) calf serum, 2.15% (v/v) chicken serum, 0.323% (v/v) NaHCO₃, 2.15% (v/v) DMSO, 1.72x glutamine, 1.72x antibiotic-antimycotic. Mix 238.3 ml H₂O, 85.7 ml 10x Ham's F10 with glutamine, 85.7 ml 2.95% (w/v) TPB, 30.1 ml calf

- serum, 10.75 ml chicken serum (heat-inactivated at 56°C for 30 min), 21.5 ml 7.5% (w/v) NaHCO₃, 10.75 ml DMSO, 8.6 ml 100x L-glutamine (200 mM), and 8.6 ml 100x antibiotic-antimycotic; store at 4°C.
23. 50x Denhardt's reagent: 1% (w/v) BSA, 1% (w/v) PVP, 1% (w/v) Ficoll 400. Dissolve 5 g BSA (bovine serum albumin), 5 g PVP (polyvinylpyrrolidone), and 5 g Ficoll 400 in H₂O to 500 ml; store in 50-ml aliquots at -20°C.
  24. Dilution buffer: 10 mM sodium phosphate pH 7.2, 187.5 mM NaCl, 1.25 % (v/v) Igepal CA-630, 1.25% (w/v) sodium deoxycholate, 2 µg/ml aprotinin. Mix 81.5 ml H₂O, 1 ml 1 M sodium phosphate pH 7.2, 3.75 ml 5 M NaCl, 1.25 ml Igepal CA-630, and 12.5 ml 10% (w/v) sodium deoxycholate; store at RT; prior to use add 1/500 vol 1 mg/ml aprotinin.
  25. D-MEM medium (-Met): D-MEM (1x, w/o: sodium pyruvate, L-Gln, L-Met, L-Cys), 5% (v/v) foetal bovine serum, 1x glutamine, 1x sodium pyruvate, 1x cysteine. Mix 46 ml D-MEM (1x w/o: sodium pyruvate, L-Gln, L-Met, L-Cys), 2.5 ml dialyzed foetal bovine serum, 0.5 ml 100x glutamine (200 mM), 0.5 ml 100x sodium pyruvate (100 mM), and 0.5 ml 100x cysteine (50 mM); store at 4°C.
  26. 1 M DTT solution: 1 M DTT, 10 mM sodium acetate pH 5.2. Dissolve 1.545 g DTT (dithiothreitol) in H₂O to 9.97 ml, add 33 µl 3 M sodium acetate pH 5.2; filter sterilize (0.22 µm); store in 1-ml aliquots at -20°C.
  27. 0.5 M EDTA. Dissolve 93.06 g EDTA (ethylenediaminetetraacetic acid) x 2 H₂O disodium salt (Titrplex III) in 400 ml H₂O; adjust pH to 8.0 with ~ 10 g NaOH; add H₂O to 500 ml; autoclave; store at RT.
  28. 5x EMSA buffer: 50 mM Tris HCl pH 7.5, 2.5 mM EDTA, 325 mM KCl, 25 mM MgCl₂, 5 mM DTT, 0.5 µg/µl BSA, 50% (v/v) glycerol. Mix 40 µl H₂O, 50 µl 1 M Tris HCl pH 7.5, 5 µl 0.5 M EDTA, 325 µl 1 M KCl, 25 µl 1 M MgCl₂, 5 µl 1 M DTT solution, 50 µl 10 mg/ml BSA, and 500 µl glycerol; store at -20°C.
  29. 1 mg/ml Ethidium bromide. Dissolve 10 mg ethidium bromide in H₂O to 10 ml; store lightprotected at RT.
  30. Focus agar: 1x Ham's F10 with glutamine, 0.295% (w/v) TPB, 3.5% (v/v) calf serum, 1.25% (v/v) chicken serum, 0.188% (v/v) NaHCO₃, 1.25% (v/v) DMSO, 1x glutamine, 1x antibiotic-antimycotic, 0.75% (w/v) bacto agar. Heat 1 vol 1.8% (w/v) bacto agar in a microwave oven; cool to 45°C in a water bath; mix with 45°C warm 1.4 vol DC3 medium; keep at 45°C in a water bath.
  31. 100x Folic acid solution: 1.81 mM folic acid, 1 M NaHCO₃. Dissolve 8.4 g NaHCO₃ in 50 ml H₂O; use this solution to dissolve 80 mg folic acid; add H₂O to 100 ml.
  32. GIEMSA staining solution. Mix 1 vol GIEMSA solution (azure-eosin-methylene blue) and 9 vol H₂O heated to 80°C; store at RT.

33. GITC buffer: 4 M guanidine thiocyanate, 25 mM sodium acetate pH 6.0, 0.835% (v/v) 2-mercaptoethanol. Dissolve 94.53 g guanidine thiocyanate in H₂O to 196.66 ml; add 1.67 ml 3 M sodium acetate pH 6.0; store at RT; prior to use add 1.67 ml 2-mercaptoethanol.
34. 1.25 M Glycine. Dissolve 4.69 g glycine in H₂O to 50 ml; filter sterilize (0.22 µm); store at 4°C.
35. Growth medium: 1x Ham's F10 with glutamine, 10% (v/v) calf serum, 0.188% (w/v) NaHCO₃, 25 mM HEPES pH 7.3, 1x antibiotic-antimycotic. Mix 370 ml H₂O, 50 ml 10x Ham's F10 with glutamine, 50 ml calf serum, 12.5 ml 7.5% (w/v) NaHCO₃, 12.5 ml 1 M HEPES pH 7.3, and 5 ml 100x antibiotic-antimycotic; store at 4°C.
36. 2x HBS (HEPES buffered saline): 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM glucose, 50 mM HEPES pH 7.05. Dissolve 6.55 g NaCl, 0.296 g KCl, 0.106 g Na₂HPO₄ x 2 H₂O, 1.19 g glucose x H₂O, and 4.77 g HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) in 360 ml H₂O; adjust to pH 7.05 with 0.5 M NaOH; add H₂O to 400 ml; filter sterilize (0.22 µm); store in 50-ml aliquots at -20°C.
37. 1 M HEPES pH 7.3 or 7.9. Dissolve 23.83 g HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) in 90 ml H₂O; adjust to pH 7.3 or 7.9 with 10 M NaOH; add H₂O to 100 ml; filter sterilize (0.22 µm); store at -20°C.
38. Hybridization solution: 50% (v/v) formamide, 6x SSC, 5x Denhardt's reagent, 5 mM EDTA, 0.2% (w/v) SDS, 100 µg/ml salmon sperm DNA. Mix 35 ml H₂O, 250 ml formamide, 150 ml 20x SSC, 50 ml 50x Denhardt's reagent, 5 ml 0.5 M EDTA, and 5 ml 20% (w/v) SDS; adjust to pH 7.4 with concentrated HCl; store light-protected at RT; prior to use boil 1/100 vol salmon sperm DNA (10 mg/ml) for 5 min; chill on ice; add to the hybridization solution.
39. 1 M KCl. Dissolve 18.64 g KCl (potassium chloride) in H₂O to 250 ml; autoclave; store at RT.
40. 1 mg/ml Leupeptin. Dissolve 5 mg leupeptin trifluoroacetate salt in H₂O to 5 ml; store in 1-ml aliquots at -20°C.
41. 5 M LiCl. Dissolve 21.2 g LiCl (lithium chloride) in H₂O to 100 ml; autoclave; store at RT.
42. Luciferase assay reagent. Contains beetle luciferin and is supplied by the Luciferase assay system E1500 (Promega); store at -80°C.
43. 1 M MgCl₂. Dissolve 20.33 g MgCl₂ (magnesium chloride) x 6 H₂O in H₂O to 100 ml; autoclave; store at RT.
44. 5 M NaCl. Dissolve 146.1 g NaCl (sodium chloride) in H₂O to 500 ml; autoclave; store at RT.

45. 1 M NaHCO₃. Dissolve 4.20 g NaHCO₃ (sodium bicarbonate) in H₂O to 50 ml; store at RT.
46. 1 M NaH₂PO₄. Dissolve 27.6 g NaH₂PO₄ (sodium dihydrogen phosphate) x H₂O in H₂O to 200 ml; autoclave; store at RT.
47. 1 M Na₂HPO₄. Dissolve 35.6 g Na₂HPO₄ (disodium hydrogen phosphate) x 2 H₂O in H₂O to 200 ml; autoclave; store at RT.
48. 2 M NaOH. Dissolve 8 g NaOH (sodium hydroxide) pellets in H₂O to 100 ml; store at RT.
49. 0.1 M NaOH, 5 mM EDTA. Mix 49 ml H₂O, 0.5 ml 10 M NaOH, and 0.5 ml 0.5 M EDTA; store at RT.
50. PBS (phosphate-buffered saline): 10 mM sodium phosphate pH 7.2, 150 mM NaCl. Mix 960 ml H₂O, 10 ml 1 M sodium phosphate pH 7.2, and 30 ml 5 M NaCl; autoclave; store at 4°C.
51. PBS-PI: 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1 mM PMSF, 2 µg/µl aprotinin, 1 µg/µl leupeptin, 1 µg/ml pepstatin A. To 100 ml PBS add 1 ml 0.1 M PMSF, 200 µl aprotinin 1 mg/ml, 100 µl leupeptin 1 mg/ml, and 100 µl pepstatin A; store at 4°C.
52. 1 mg/ml Pepstatin. Dissolve 5 mg pepstatin A in DMSO to 5 ml; store in 1-ml aliquots at -20°C.
53. PGM (primary growth medium): 1x Ham's F10 with glutamine, 0.295% (w/v) TPB, 8% (v/v) calf serum, 2% (v/v) chicken serum, 0.188% (w/v) NaHCO₃, 1x antibiotic-antimycotic. Mix 332.5 ml H₂O, 50 ml 10x Ham's F10 with glutamine, 50 ml 2.95% (w/v) TPB, 40 ml calf serum, 10 ml chicken serum (heat-inactivated at 56°C for 30 min), 12.5 ml 7.5% (w/v) NaHCO₃, and 5 ml 100x antibiotic-antimycotic; store at 4°C.
54. 0.1 M PMSF. Dissolve 174 mg PMSF (phenylmethylsulfonyl fluoride) in isopropanol to 10 ml; store in 1-ml aliquots at -20°C.
55. Proteinase K solution: 10 mg/ml proteinase K. Dissolve 100 mg proteinase K (10 mg/ml) in H₂O to 10 ml; store in 1-ml aliquots at -20°C.
56. RIPA buffer: 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% (v/v) Igepal CA-630, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2 µg/ml aprotinin. Mix 422.5 ml H₂O, 5 ml 1 M sodium phosphate pH 7.2, 15 ml 5 M NaCl, 5 ml Igepal CA-630, 50 ml 10% (w/v) sodium deoxycholate, and 2.5 ml 20% (w/v) SDS; filter sterilize (0.22 µm); store at 4°C; prior to use add 1/500 vol aprotinin 1 mg/ml.
57. RIPA-BSA: 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% (v/v) Igepal CA-630, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2 µg/ml aprotinin, 2 mg/ml BSA. Dissolve 100 mg BSA in RIPA buffer to 50 ml; store at 4°C; prior to use add 1/500 vol aprotinin 1 mg/ml.

58. RIPA-protein A-bead slurry: 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% (v/v) Igepal CA-630, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10% (v/v) protein A sepharose CL-4B. Wash 100 mg protein A sepharose CL-4B beads 3 x with each 20 ml H₂O (during the washing the beads should swell on ice for 30 min); determine the volume of the swollen beads; add 9 vol of RIPA buffer; store at 4°C.
59. RIPA-sucrose: 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% (v/v) Igepal CA-630, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2 µg/ml aprotinin, 10% (w/v) sucrose. Dissolve 10 g sucrose in RIPA buffer to 100 ml; filter sterilize (0.22 µm); store at 4°C; prior to use add 1/500 vol aprotinin 1 mg/ml.
60. RNA buffer A: 1 M NaCl, 40 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1% (w/v) SDS. Mix 150.6 ml H₂O, 40 ml 5 M NaCl, 8 ml 1 M Tris HCl pH 7.5, and 400 µl 0.5 M EDTA; autoclave; add 1 ml 20% (w/v) SDS; store at RT.
61. RNA buffer B: 0.1 M NaCl, 20 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1% (w/v) SDS. Mix 190.6 ml H₂O, 4 ml 5 M NaCl, 4 ml 1 M Tris HCl pH 7.5, and 400 µl 0.5 M EDTA; autoclave; add 1 ml 20% (w/v) SDS; store at RT.
62. RNA buffer C: 10 mM Tris HCl pH 7.5, 1 mM EDTA, 0.05% (w/v) SDS. Mix 197.1 H₂O, 2 ml 1 M Tris HCl pH 7.5, and 400 µl 0.5 M EDTA; autoclave; add 0.5 ml 20% (w/v) SDS; store at RT.
63. 10x RNA gel running buffer: 200 mM MOPS, 80 mM sodium acetate, 10 mM EDTA. Dissolve 41.2 g MOPS [3-(N-morpholino)-propanesulfonic acid] in ~ 800 ml H₂O; add 26.6 ml 3 M sodium acetate pH 5.2, and 20 ml 0.5 M EDTA; adjust to pH 7.0 with 2 M NaOH; add H₂O to 1000 ml; store light-protected at 4°C.
64. RNA sample buffer: 6% (v/v) formaldehyde, 70% (v/v) formamide, 0.03% (w/v) bromophenol blue, 1x RNA gel running buffer, 1% (w/v) Ficoll 400. Dissolve 0.1 g Ficoll 400 in 1 ml 10x RNA gel running buffer, 0.3 ml 1% (w/v) bromophenol blue, 1.65 ml formaldehyde solution (36.5%), and 7 ml formamide; store in 1-ml aliquots at -20°C.
65. 20% (w/v) SDS. Dissolve 20 g SDS (sodium dodecylsulfate) in H₂O to 100 ml; store at RT.
66. 1x SDS gel-loading buffer: 60 mM Tris HCl pH 6.8, 3% (w/v) SDS, 10% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 5% (v/v) 2 mercaptoethanol. Mix 6.35 ml H₂O, 0.6 ml 1 M Tris HCl pH 6.8, 1.5 ml 20% (w/v) SDS, 1 ml glycerol, and 50 µl 1% (w/v) bromophenol blue; store at RT; prior to use add 1/20 vol 2-mercaptoethanol.
67. SDS lysis buffer: 1% (w/v) SDS, 10 mM EDTA, 50 mM Tris HCl pH 8.0, 1 mM PMSF, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A. Mix 44 ml H₂O, 2.5 ml 20% (w/v) SDS, 1 ml 0.5 M EDTA, and 2.5 ml 1 M Tris HCl pH 8.0; prior to use add 1/100 vol 0.1 M PMSF, 1/500 vol aprotinin 1 mg/ml, 1/1000

- vol leupeptin 1 mg/ml, and 1/1000 vol pepstatin A 1 mg/ml; filter sterilize (0.22 μ m); store at RT.
68. 1.5% (w/v) Sea plaque agarose. Suspend 1.5 g sea plaque agarose in 100 ml H₂O; autoclave; store at 4°C.
  69. Shock solution: 15% (v/v) glycerol in buffered F10 medium. Mix 85 ml buffered F10 medium and 15 ml glycerol; prepare fresh; prior to use warm to 37°C.
  70. 3 M Sodium acetate pH 5.2 or 6.0. Dissolve 24.61 g sodium acetate in 80 ml H₂O; adjust to pH 5.2 or 6.0 with acetic acid; add H₂O to 100 ml; autoclave; store at RT.
  71. 1 M Sodium phosphate pH 7.2. Mix 136.8 ml 1 M Na₂HPO₄ and 63.2 ml 1 M NaH₂PO₄; check pH; autoclave; store at RT.
  72. 20x SSC: 3 M NaCl, 0.3 M sodium citrate. Dissolve 175 g NaCl and 88 g sodium citrate in H₂O to 1000 ml; autoclave; store at RT.
  73. Stripping solution: 5 mM Tris HCl pH 8.0, 0.1x Denhardt's reagent, 2 mM EDTA. Mix 989 ml H₂O, 5 ml 1 M Tris HCl pH 8.0, 2 ml 50x Denhardt's reagent, and 4 ml 0.5 M EDTA; store at 4°C.
  74. 10x T4 PNK buffer: 700 mM Tris HCl pH 7.5, 100 mM MgCl₂, 50 mM DTT. Mix 150 μ l H₂O, 700 μ l 1 M Tris HCl pH 7.5, 100 μ l 1 M MgCl₂, and 50 μ l 1 M DTT solution; store at -20°C.
  75. 10x TBE: 890 mM Tris, 890 mM boric acid, 20 mM EDTA. Dissolve 108 g Tris base and 55 g boric acid in H₂O to 960 ml; add 40 ml 0.5 M EDTA; autoclave; store at RT.
  76. TE buffer: 10 mM Tris HCl pH 8.0, 1 mM EDTA. Mix 988 ml H₂O, 10 ml 1 M Tris HCl pH 8.0, and 2 ml 0.5 M EDTA; autoclave; store at RT.
  77. 2.95% (w/v) TPB. Dissolve 29.5 g TPB (tryptose phosphate broth) in H₂O to 1000 ml; autoclave; store at 4°C.
  78. Transfer buffer: 10% (v/v) calf serum in TS buffer. Mix 10 ml calf serum and 90 ml TS buffer; store at 4°C.
  79. 1 M Tris HCl pH 6.8 or 7.5 or 8.0. Dissolve 60.57 g Tris base in 400 ml H₂O; adjust to pH 6.8 or 7.5 or 8.0 with concentrated HCl; add H₂O to 500 ml; autoclave; store at RT.
  80. Trypsin-EDTA solution: 1x Trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA) in TS buffer. Mix 5 ml 10x Trypsin-EDTA and 45 ml TS buffer; store at 4°C.
  81. TR solution: 5x Trypsin-EDTA in TS buffer. Mix 7.5 ml 10x Trypsin-EDTA and 7.5 ml TS buffer; store at 4°C.
  82. TS (Tris saline) buffer: 150 mM NaCl, 5 mM KCl, 25 mM Tris HCl pH 7.5, 5 mM glucose. Dissolve 0.99 g glucose x H₂O in ~ 200 ml H₂O, add 30 ml 5 M NaCl, 5 ml 1 M KCl, 25 ml 1 M Tris HCl 7.5, and H₂O to 1000 ml; autoclave; store at 4°C.

83. Washing solution 2x SSC: 2x SSC, 0.1% (w/v) SDS, 1 mM EDTA. Mix 893 ml H₂O, 100 ml 20x SSC, 5 ml 20% (w/v) SDS, and 2 ml 0.5 M EDTA; store at RT.
84. Washing solution 0.2x SSC: 0.2 x SSC, 0.1% (w/v) SDS, 1 mM EDTA. Mix 983 ml H₂O, 10 ml 20x SSC, 5 ml 20% (w/v) SDS, and 2 ml 0.5 M EDTA; store at RT.

3 Methods

3.1 Preparation and cultivation of primary quail embryo fibroblasts (QEF)

1. Incubate fertilized quail eggs for 9 d at 37.8°C in an egg incubator with a water-saturated atmosphere (>60% humidity) and automatic turning device.
2. Clean the egg with ethanol and open at the shallow site using a spatula.
3. Remove the broken shell with a sterile forceps, then the membrane under the egg shell with another sterile forceps.
4. Gently pull out the embryo with closed forceps under its neck.
5. Put the embryo onto a petri dish. Remove head, arms, legs and transfer the body into a 50 ml tube.
6. Wash the body with 10 ml *TS buffer* (37°C), shake briefly, decant the supernatant and thoroughly homogenize the body with the round end of a spatula for 5 min.
7. Add 10 ml *TR solution* (37°C), shake gently, incubate for 5 minutes at room temperature (RT), then carefully pipett 5 x up and down using a 10 ml wide mouth pipette.
8. Let clumps settle down for 5 min, then decant the supernatant into a prepared 50 ml tube containing 10 ml ice-cold *PGM* (see Note 1).
9. Repeat this washing step with following solutions kept at 37°C: 5 ml *TS buffer*, 5 ml *TS buffer*, 5 ml *TR solution*, 5 ml *TS buffer*, 5 ml *TS buffer*.
10. Finally, mix cell suspension by inverting the 50 ml tube with the cells and centrifuge at 150 x g for 20 min at 4°C.
11. Decant the supernatant, add 20 ml *PGM* to the pellet and pipett 25 x up and down with a wide mouth pipette.
12. Let the clumps settle down for 5 min and transfer the supernatant into a fresh 50 ml tube.
13. From this supernatant remove 0.5 ml and dilute to 5 ml with *TS buffer* for determination of the cell number.
14. Rinse a hemocytometer and its cover slip with ethanol, then put the cover slip onto the chamber.
15. Pipett one drop of the diluted cell suspension at the edge of the cover slip and put the device onto a microscope.

16. Count the cells except the dark and smooth blood cells in the four fields encompassing 16 squares (0.1 mm^3) each and determine the average cell number of one field [calculation of the cell number/ml: N (average number of cells in one field) $\times 10$ (dilution factor) $\times 10^4$]. The total number of cells should be in the range of 10^8 for a 9 day old embryo.
17. Plate 6×10^6 cells per 100-mm dish containing 10 ml *PGM*.
18. Incubate cells at 37°C in a 5% CO_2 humidified atmosphere and replace the medium after three days with 12 ml *PGM* per dish.
19. Culture the cells at 37°C (5% CO_2) in a water-saturated atmosphere.
20. For transfer, wash the cells of one dish with 5 ml *TS buffer*, aspirate, add 2 ml *trypsin-EDTA solution*, and aspirate.
21. Incubate at 37°C for 5-10 min (see Note 2), then suspend the cells in 2 ml of *transfer buffer* and transfer into a conical 15-ml tube.
22. Wash the dish with 3 ml of culture medium and add to the cell suspension.
23. From the final volume of 5 ml dilute 0.1 ml with 9.9 ml of ISOTON™ II Diluent.
24. Count the cells using a Coulter counter, determine the number of cells/ml and the total cell number of the trypsinized dish.
25. Calculate the volume of cell suspension ($3\text{--}6 \times 10^6$) to be seeded into 100-mm dishes containing 10 ml of *avian cell culture medium*.
26. Change the medium after 2-3 days.

3.2 Infection of QEF with *myc*-carrying retroviruses

1. Calculate the amount of QEFs needed and remove medium from an appropriate number of dishes.
2. Wash the cells of each dish with 5 ml *TS buffer*, aspirate, add 2 ml *trypsin-EDTA solution*, and aspirate.
3. Incubate at 37°C for 5-10 min, then suspend the cells in 2 ml *transfer buffer* per dish and transfer into a conical tube.
4. Wash each dish with 3 ml of *growth medium* and add to the cell suspension.
5. Dilute 0.1 ml of the cell suspension with 9.9 ml of Isoton® II Diluent.
6. Count the cells using a Coulter counter and determine the number of cells/ml.
7. Dilute the cell suspension with *growth medium* to a concentration of 3×10^6 cells/ml.
8. Seed 1 ml of this suspension into a 100-mm dish containing 10 ml of *growth medium* and incubate at 37°C (5% CO_2) for 30 min.
9. Meanwhile quickly thaw a viral MC29 stock in a water bath at 37°C (see Note 3).

10. Add 1 ml of the viral stock to the cells and incubate over night at 37°C (5% CO₂).
11. The next day aspirate the culture medium from the cells and replace with 10 ml of *avian cell culture medium*.
12. Cultivate the cells for two weeks to allow a complete spread of the virus.
13. To collect MC29 virus, remove the 2-3 days old culture medium from confluent grown cells and freeze at -80°C.

3.3 DNA transfection of *myc*-carrying retroviral vector plasmids

1. Prepare a cell suspension from avian fibroblasts as described above.
2. Dilute the cell suspension with *growth medium* to a concentration of 1.25×10^6 cells/ml and seed each 1 ml of the cell suspension into 60-mm dishes containing 4 ml of *growth medium* (see Note 4).
3. Incubate over night at 37°C (5% CO₂). The cells should be almost confluent the next day.
4. Before transfection replace the medium with fresh *growth medium* and incubate at 37°C for 1 h (5% CO₂).
5. To prepare the transfection cocktail add 50 µl of 2.5 M CaCl₂ to 450 µl of H₂O containing 5 µg of cesium chloride-purified plasmid DNA. Drop this mixture into a conical tube containing 0.5 ml of 2x HBS. Vortex at 1,800 rpm for 2 s.
6. After the solution has become turbid due to calcium phosphate precipitation, slowly drop the transfection mixture into the medium of the seeded cells.
7. Gently swirl the dishes and incubate at 37°C for 4 h.
8. Remove the medium and add 2 ml of 37°C warm *shock solution* (see Note 5).
9. Incubate for 2 min at RT then add 5 ml of *buffered F10 medium*.
10. Aspirate, then add 5 ml of *buffered F10 medium*.
11. Aspirate, then add 5 ml of *avian cell culture medium* and incubate the cells at 37°C (5% CO₂).

3.4 Focus assay of *myc*-transformed cells

1. Transfect cells seeded on 60-mm dishes with varying amounts of plasmid DNA (0.5 µg - 5.0 µg) using the calcium phosphate method, or infect cells grown on 60-mm dishes with 300 µl of serial dilutions (10⁰ to 10⁻⁵) from a viral MC29 stock (see Note 6).
2. After transfection or infection cultivate the cells in 5 ml of *growth medium* at 37°C (5% CO₂).
3. The next day remove the medium from the confluent cells and add 4 ml of liquid *focus agar* (see Note 7).

4. Let the agar harden for 1 h at RT, then incubate the cells at 37°C (5% CO₂).
5. Overlay cells every 2-3 d with 2 ml of *focus agar*. Foci of transformed cells are detectable after 1-2 weeks.
6. To pick single foci, prepare a flexible plastic tube attached to a Pasteur pipette and MP-24 wells filled with each 1 ml of *avian cell culture medium* (see Note 8).
7. Under the microscope detach the focus and carefully suck until the focus is visible in the pipette tip (see Note 9).
8. Transfer the focus into the MP-24 well and rinse the pipette with culture medium.
9. Incubate at 37°C (5% CO₂) for 1 d until cells have been attached to the dish. Replace medium.
10. After cells have grown to confluence, transfer cells from the entire well into an MP 6 well, then to a 60-mm dish, and finally to a 100-mm dish to propagate cells derived from a single focus.
11. For scoring the number of foci, remove the agar block from the cells and wash twice with *TS buffer*.
12. Add 5 ml of absolute ethanol to fix the cells. Incubate for 2 min at RT.
13. Aspirate ethanol, cover the cells with 2 ml of *GIEMSA staining solution*, and incubate for 30 min at RT (do not rock the dishes).
14. Remove the staining solution and wash 3 x with water. Air-dry the dishes and score foci by counting and photography (see Fig. 1).
15. Determine the number of foci per microgram of transfected DNA to quantify the transformation efficiency.

3.5 Colony assay of *myc*-transformed cells

1. Transfect or infect cells plated on 60-mm dishes as described above.
2. 2-3 d after transfection or infection transfer the cells into a 100-mm dish.
3. Cultivate the cells by transferring them every 4-7 d until they have adopted a transformed morphology (3-6 passages).
4. Prepare MP-6 dishes filled with each 1 ml of liquid *cloning bottom agarose* per well. Let the agar harden for 30 min (see Note 7).
5. Prepare an adequate amount of *cloning top agarose* and keep at 45°C in a water bath.
6. Transfer trypsinized cells suspended in 5 ml of *transfer buffer* into a conical tube.
7. Determine the cell number and transfer each 10,000 to 100,000 cells into 3-ml tubes.

8. Add each 2 ml of liquid *cloning top agarose* (45°C) into the tubes and mix with a 2-ml wide-mouth pipette.
9. Pour the mixtures into the prepared MP-6 wells. Let the agar harden for 2 h at RT, then incubate at 37°C (5% CO₂).
10. Overlay the cells every 2-3 d with 1 ml of *cloning top agarose* per well (see Note 10). Colonies consisting of transformed cells are detectable after 1-2 weeks (see Fig. 1).
11. Determine the number of colonies formed by 1,000 cells to quantify the transformation efficiency.
12. To generate cell clones derived from single colonies, prepare a flexible plastic tube attached to a Pasteur pipette and MP-24 wells filled with each 1 ml of *avian cell culture medium*.
13. Localize the colony under the microscope and carefully suck until the colony is visible in the pipette.
14. Hold the pipette into the MP-24 well and rinse with culture medium until the colony has been transferred into the well.
15. Incubate at 37°C (5% CO₂) for 1 d until cells have been attached to the dish. Replace medium.
16. After cells have grown to confluence, transfer cells from the entire well to an MP-6 well, then to a 60-mm dish, and finally to a 100-mm dish to propagate cells derived from a single agar colony.

3.6 Northern analysis of *c-myc* or *v-myc* RNAs

1. Grow the cells to near confluence and change the medium one day prior to RNA isolation.
2. Aspirate the culture medium with a Pasteur pipette attached to a water pump and wash cells with 5 ml of *TS buffer* per plate.
3. Aspirate and place the plates in tilted position to remove residual liquid.
4. Spread 2-4 ml *GITC buffer* per plate onto the cells and agitate plates on a rocking platform to distribute the buffer and to detach the lysed cells.
5. Put the plates in a tilted position and transfer the lysate into a plastic tube.
6. Draw the lysate three times through a 20-gauge needle to shear the chromosomal DNA.
7. Transfer 4 ml of *CsCl buffer* into an ultracentrifuge polyallomer tube.
8. Carefully layer 7.5-8 ml of cell lysate onto the CsCl-cushion without mixing both phases (consider the maximal loading capacity per ultracentrifuge tube which is lysate from 10⁸ cells) and fill up the tube to 2 mm from the top. Balance the tubes with *GITC buffer*.

9. Insert the tubes into the rotor buckets (6) and run samples at 25,000 rpm (SW41 rotor) at 21°C for 16-24 h.
10. After the run has been completed, remove both phases by pipetting until approximately 1 ml liquid remains at the bottom of the tube (*see Note 11*).
11. Quickly invert the tube and let drain all liquid onto a paper towel.
12. With a sterile scalpel cut off the bottom of the tube.
13. Dissolve the RNA pellet in 2 x 200 µl 0.3 M sodium acetate pH 6.0 for 3 min each and transfer into a microcentrifuge tube (*see Note 12*).
14. Add 1 ml of absolute ethanol, store at -80°C for 30 min and centrifuge at 13,000 x g for 15 min at 4°C.
15. Wash the pellet in 70% ethanol and dry in a Speed-Vac (do not dry to completion!).
16. Dissolve the RNA in 100 µl H₂O per 10⁸ cells. Store the RNA solution at -80°C.
17. Determine the optical densities (OD) at 260 and 280 nm; for pure RNA the OD₂₆₀:OD₂₈₀ ratio is 2.0.
18. Calculate the RNA concentration by using the correlation 1 OD₂₆₀ = 40 µg/ml. Yields vary between 0.5 mg and 2 mg of total RNA per 10⁸ cells.
19. For poly(A)⁺-RNA selection fill 100-200 mg oligo dT-powder (for 0.5-5 mg of total RNA) into a 10 ml tube and suspend by inverting several times in 5 ml of *RNA buffer B*.
20. After 10 minutes equilibration fill the suspension into a sterile Econo-Column chromatography column (0.7 x 5 cm; 2 ml).
21. Rinse the column with 4 ml of 0.1 M NaOH, 5 mM EDTA.
22. Wash the column with H₂O until the pH becomes neutral (check with pH paper).
23. Equilibrate the column with 5 ml of *RNA buffer A*.
24. Meanwhile heat the total RNA in a volume of 500 µl at 65°C for 5 min.
25. Add 500 µl *RNA buffer A* (65°C) to the RNA, mix, and leave at RT for 2 min.
26. Load the RNA solution onto the column, collect the eluate and heat again at 65°C for 5 min.
27. Reload the RNA as before.
28. Wash the column 5 times with 1 ml of *RNA buffer B*.
29. Elute the poly(A)⁺ RNA with 2 ml of *RNA buffer C*.
30. For a second round of poly(A)⁺ RNA selection use a column with 50 mg of oligo dT-powder and repeat the procedure as described above.
31. Elute the poly(A)⁺ selected RNA with 4 x 450 µl *buffer C* into four microcentrifuge tubes.

32. To each tube add 50 μ l 3 M sodium acetate pH 6.0 and 1 ml absolute ethanol, mix, and store at -80°C for 30 min.
33. Spin at 13,000 x g for 20 min at 4°C .
34. Wash the pellets with 70% ethanol and centrifuge as above.
35. Dry the pellets in a Speed-Vac and dissolve the poly(A)⁺ RNA in the appropriate amount of H₂O to achieve a concentration of $\sim 1 \mu\text{g}/\mu\text{l}$. Store the RNA solution at 80°C .
36. Determine the the RNA concentration. The yield after the first selection should be 5-10% from the total RNA (10-20 fold enrichment) and 1-2% after the second selection (50-100 fold enrichment).
37. For gel electrophoresis of isolated RNAs pour a 1% (w/v) agarose/formaldehyde gel and let the gel harden for at least 1 h.
38. To 4-8 μ l of RNA (30 μg total RNA or 1-5 μg poly(A)⁺-selected RNA dissolved in 4-8 μ l H₂O) add 16 μ l of RNA sample buffer.
39. Heat samples to 65°C for 10 min, centrifuge briefly, and then add 1 μ l of 1 mg/ml ethidium bromide.
40. Load the samples onto the gel and run at 30-40 V const. overnight (500 Vh) in 1x RNA gel running buffer until the bromophenol blue dye has migrated ~ 10 cm.
41. Photograph the gel with an aligned ruler next to the size marker and cut the gel to an appropriate size (e.g. 15 cm x 12 cm).
42. Rinse the gel in deionized water for 5 min to remove the formaldehyde.
43. Equilibrate the gel in 10x SSC for 30 min.
44. Prepare a nylon membrane sheet of the appropriate size and equilibrate first in sterile water for 10 min, then in 10x SSC for 30 min.
45. With a tray and a board prepare a blotting device and fill the tray with 10x SSC.
46. Cut two sheets of Whatman^{TM} paper (20 cm x 30 cm), soak them in 10x SSC and create a wick to support the gel.
47. Put the inverted gel onto the device and insulate the area around the gel with Parafilm^{TM}.
48. Layer the nylon membrane onto the gel and remove air bubbles with a pipette.
49. Layer three pieces of Whatman^{TM} paper (cut to the gel size and soaked in 10x SSC) onto the membrane and remove air bubbles.
50. Layer paper towels onto the Whatman^{TM} papers and put a 0.5-1 kg-weight onto the top.
51. Transfer 16-24 h.

52. After the transfer, mark positions of the slots with a ball-pen and put the membrane into 500 ml of *2x SSC* for 10 min to remove rests of agarose.
53. Bake the membrane wrapped between two sheets of Whatman™ paper in a vacuum oven for 2 h at 80°C.
54. After baking, wrap the blot in aluminum foil and store dark and dry.
55. For detection of transferred RNAs by nucleic acid hybridization, pre-hybridize the filter in 100 ml *hybridization solution* in a plastic tray over night at 37°C.
56. Boil a radioactive labeled DNA probe (pierce a hole into the tube cap) for 5 min in a water bath for denaturation and then chill on ice.
57. After pre-hybridization transfer the filter into a new tray containing 40 ml *hybridization solution* and the denatured radiolabeled probe.
58. Hybridize the filter over night at 37°C.
59. The next day wash the filter three times for 10 min each in *washing solution 2x SSC* at 37°C.
60. Then wash the filter three times for 20 min each in *washing solution 0.2x SSC* at 60°C.
61. Let the filter dry on a Whatman™ paper. Then wrap into a plastic foil and autoradiograph at -80°C using an intensifier screen (see Fig. 2).
62. To remove the hybridized probe from the blot, incubate the filter sequentially for 1 h at 65°C and 1 h at 70°C in *stripping solution*.

3.7 Immunoprecipitation of Myc proteins

1. Seed 2.5×10^6 cells onto 60-mm dishes and incubate in *avian cell culture medium* for one day at 37°C and 5% CO₂.
2. Wash the cells twice with 2.5 ml PBS and once with 1 ml D-MEM.
3. Incubate the cells for 1 h at 37°C (5 % CO₂) in 2.5 ml *D-MEM medium (-Met)*.
4. Remove the medium by aspiration and replace it with 1 ml *D-MEM medium (-Met)* containing 50 µCi ³⁵S-methionine (>1000 Ci/mmol) per dish.
5. Incubate the dishes for 2-3 h at 37°C (5% CO₂) with occasional rocking.
6. Wash the cells twice with 2.5 ml ice-cold *PBS*, aspirate and drain the last traces.
7. Place the dishes onto a surface chilled at 0°C.
8. Scrape the cells of one 60-mm dish into 250 µl *boiling buffer* and transfer into a 1.5-ml reaction tube (see Note 13).
9. Pierce a hole into the cap and heat for 2 min at 100°C in a boiling water bath, then cool on ice for 15 min.
10. Dilute the lysate with 1 ml *dilution buffer* and pipett up and down several times to shear chromosomal DNA.

11. Centrifuge at 20,000 x g for 60 min at 4°C.
12. Transfer the clarified lysates into cooled fresh tubes and determine the activity from 6.25 µl (1/200 vol) dissolved in 10 ml 10 ml ReadyMix™ (Beckmann) using a scintillation counter.
13. Cell extracts may be stored at this stage at -80°C.
14. For each precipitate prepare 1.5-ml reaction tubes containing 1-5 µl of antiserum or pre-immune serum diluted in 200 µl *RIPA-BSA*.
15. Dilute clarified lysate corresponding to 1-2 x 10⁷ cpm to a volume of 500 µl with *RIPA buffer* and mix with the diluted antiserum.
16. Incubate on ice for 1-2 h.
17. Add 100 µl of *RIPA-protein A-bead slurry* and incubate for 60 min at 4°C on a rocking platform.
18. Pellet the immune complexes by centrifugation at 20,000 x g for 20 s at 4°C.
19. Discard the supernatant and resuspend the pellets in 650 µl *RIPA buffer*.
20. Transfer this suspension onto a cushion of 650 µl *RIPA-sucrose*.
21. Centrifuge at 13,000 x g for 15 min at 4°C.
22. Discard the supernatant and wash the pellets 3 x with 1 ml *RIPA buffer*, and once with 1 ml *PBS*.
23. After the last wash suspend the pellet in 40 µl *1x SDS gel-loading buffer* and incubate at 95°C for 5 min.
24. Centrifuge at 15,000 x g for 5 min at RT.
25. Load the supernatant (*see* Note 14) directly onto a SDS polyacrylamide gel (*see* Fig. 1).

3.8 Analysis of Myc-DNA interactions by electrophoretic mobility shift assay (EMSA)

1. To prepare a radiolabeled double-stranded DNA probe mix 100 pmol each of two single-stranded complementary oligonucleotides (18-22mers), 5 µl *10x annealing buffer*, and H₂O to 50 µl (DNA concentration: ~ 25 ng/µl).
2. Incubate at 95-100°C in a boiling water bath for 3 min, and then switch off the heating plate.
3. Leave until the reaction mix has cooled down to RT (~ 3 h). Store at -20°C.
4. To 75 ng of the annealed DNA add 1 µl *10x T4 PNK buffer*, 3 µl ³²P-γ-ATP (3,000 Ci/mmol), H₂O to 9 µl, and 1 µl of T4 DNA polynucleotide kinase.
5. Incubate at 37°C for 1 h.
6. Add 30 µl H₂O, 10 µl *5 M LiCl*, 200 µl absolute ethanol, mix and store at -80°C for 1 h.

7. Collect the precipitated oligonucleotide by centrifugation at 15,000 x g for 30 min at 4°C.
8. Remove the supernatant and wash the pellet with 1 ml of 70% (v/v) ethanol. Recover the DNA by centrifugation as above.
9. Remove the supernatant and dry the pellet in a Speed-Vac for 5 min at RT.
10. Dissolve the pellet in 50 µl *1x annealing buffer* and determine the γ -HCH activity from 1/100 vol. An activity of 0.5-1.0 x 10⁸ cpm/µg DNA should be obtained. Store the DNA probe at -20°C.
11. For the EMSA reaction dilute the DNA probe to 10,000 cpm/µl with *1x EMSA buffer* resulting into a DNA concentration of 7.5 nM (~ 0.1 ng/µl).
12. Determine the protein concentration and the molarity of the recombinant protein (e.g. a 1 µg/µl solution of a protein with a M_r of 20,000 is 50 µM).
13. Dilute the recombinant protein solution with *1x EMSA buffer* to a concentration of 1 µM (20 ng/µl for a protein with M_r of 20,000).
14. To 5 µl protein solution add 17.5 µl *1x EMSA buffer*, and 2.5 µl DNA probe (final concentrations: protein, 200 nM; DNA, 0.75 nM).
15. To titer the optimal amount of protein which is required to consume all DNA, set up the same binding reaction using serial 1:2 dilutions of the 1-µM protein stock solution resulting in final concentrations of 100 nM, 50 nM, 25 nM etc.
16. Incubate the reactions (final volume: 25 µl) at 25°C for 45 min.
17. To detect protein-DNA interactions, perform gel electrophoresis as described below.
18. The protein concentration [P] which is necessary to bind to 50% of the applied DNA probe is equivalent to the dissociation constant (K_d) according to $K_d = [P] \times [DNA] / [P-DNA]$.
19. To specify the binding protein, an appropriate antibody (0.5-1 µl) may be included in the reaction (see Note 15).
20. For detection of DNA binding by gel electrophoresis pour a 5% (w/v) polyacrylamide gel, mix 31,3 ml H₂O with 6,7 ml 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide, 2 ml 10x TBE, 250 µl 10% (w/v) APS, and 35 µl TEMED (N,N,N',N'-tetramethylethylenediamine).
21. Cast a vertical gel using 1.5-mm spacers and let polymerize for 1 h.
22. Remove the comb; rinse the slots with 0.5x TBE, and insert the glass plates with the gel into an appropriate gel chamber. As running buffer use 0.5x TBE.
23. Load the samples using a Hamilton syringe. Into an extra slot fill 25 µl *1x EMSA buffer* containing 0.1% (w/v) bromophenol blue.
24. Electrophorese at constant 20 mA for approximately 2 h until the bromophenol blue dye has passed 2/3 of the gel.

25. Rock the gel for 15 min in 10% (v/v) acetic acid at RT (*see* Note 16).
26. Put the inverted gel onto a glass plate and drain excess fluid.
27. Layer two sheets of Whatman™ 3MM CHR paper onto the gel and dry for 75 min at 65°C on a gel dryer.
28. Wrap the gel into a plastic foil and autoradiograph at -80°C using an intensifier screen for 2-16 h (*see* Fig. 3). For quantification of radioactive signals, use a phosphorimager.

3.9 Analysis of Myc-induced transcriptional activation by reporter gene assay

1. Co-transfect cells in triplicate seeded on 60-mm dishes with appropriate expression and reporter plasmids (total 5 µg).
2. After 1-2 d wash cells with 2.5 ml *PBS buffer* per dish and aspirate.
3. Add 250 µl *1x cell culture lysis reagent* per dish (*see* Note 17).
4. Place the dishes onto an ice-cooled sheet of aluminum foil and rock for 3 min.
5. Scrape the cells using a rubber policeman and transfer into a 1.5-ml reaction tube.
6. Vortex the tube for 15 s, then centrifuge at 4°C for 10 min at 15,000 x g.
7. Transfer the supernatant into a fresh tube and keep on ice. Cell extracts may be stored at this stage at -80°C.
8. Determine the protein concentration (e.g. by Bradford assay).
9. Thaw the *luciferase assay reagent* and keep at 25°C (*see* Note 18).
10. Program a luminometer (e.g. LUMAT LB 9507, Berthold Technologies) to perform a 10 s-measurement.
11. Dispense 5 µl of the cell lysate into a luminometer tube and add 40 µl *luciferase assay reagent*.
12. Put the tube into the luminometer and initiate reading.

3.10 Analysis of Myc binding to target promoters by ChIP assay

1. Use cells from three 100-mm dishes grown to subconfluency (~ 10⁷ cells/dish). Determine the cell number from an extra dish.
2. Remove the culture medium, add 9.725 ml of fresh culture medium and 275 µl of 36.5% (v/v) formaldehyde to a final concentration of 1% (v/v).
3. Incubate at RT for 15 min.
4. Add 1 ml of 1.25 M *glycine* and incubate at RT for 5 min.
5. Remove the culture medium and wash 2 x with ice cold *PBS*.
6. Add 2 ml of ice cold *PBS-PI* and incubate on ice for 3 min on a rocking platform.

7. Scrape the cells and transfer the cell suspension into a conical tube. Wash the dish with each 4 ml of *PBS-PI*.
8. Pellet the cells by centrifugation at 720 x g for 10 min at 4°C.
9. Resuspend the cells in 6 ml *PBS-PI* and centrifuge as above.
10. Add 0.33 ml of *SDS-lysis buffer* per 10⁷ cells and homogenize. Incubate on ice for 10 min.
11. Transfer the lysate (~ 1 ml) into a 10-ml reaction tube and sonicate at constant 40 W with 25% power for 10 s (Branson sonifier) (see Note 19).
12. Repeat the sonication 3-5 x keeping the samples and the sonifier tip cooled. The sheared chromatin should have DNA sizes between 0.2 and 1 kbp (see below) (see Note 20).
13. Centrifuge the extract at 15,000 x g for 10 min at 4°C to pellet cell debris.
14. Save the supernatant (undiluted extract) which can be stored at -80°C.
15. To monitor sonication mix 4 µl of 5 M NaCl with 100 µl of undiluted extract and incubate at 65°C for 4 h. Recover the liberated DNA by phenol/chloroform extraction and analyze 10-µl, 5-µl, and 2-µl aliquots from the aqueous phase by agarose gel electrophoresis.
16. For immunoprecipitation dilute the supernatant (~ 0.9 ml) in a 10-fold volume of *ChIP-dilution buffer*.
17. To 40 µl of the diluted extract add 410 µl *ChIP-elution buffer* and store at -20°C (input sample).
18. For pre-clearing the lysate, add 75 µl of *ChIP-protein A-bead slurry* to 1 ml of the sheared and diluted chromatin extract. Incubate at 4°C for 1 h using a rotary shaker.
19. Centrifuge at 1,500 x g for 5 min at 4°C.
20. Transfer the pre-cleared supernatant into a fresh 1.5-ml reaction tube and add 2 µl of specific antiserum. Include each one reaction with pre-immune serum and without antiserum as a control.
21. Incubate overnight at 4°C using a rotary shaker.
22. Add 75 µl of *ChIP-protein A-bead slurry* and incubate at 4°C for 1 h using a rotary shaker.
23. Centrifuge at 1,500 x g for 5 min at 4°C.
24. Discard the supernatant and add 1 ml of *ChIP-low salt wash buffer* to the pellet.
25. Incubate at 4°C for 5 min using a rotary shaker and centrifuge as above.
26. Wash as above with 1 ml of *ChIP-high salt wash buffer*.
27. Wash as above with 1 ml of *ChIP-LiCl wash buffer*.

28. Wash 2 x with each 1 ml of *TE buffer*.
29. To the pellet add 225 µl of *ChIP-elution buffer* and incubate at for 15 min at RT using a rotary shaker.
30. Centrifuge at 1,500 x g for 5 min at 4°C and recover the supernatant containing the cross-linked chromatin complexes.
31. Repeat the elution and then pool the supernatants resulting in a final volume of 450 µl.
32. Dissociate the cross-linked material (including from the Input sample) by adding 18 µl of 5 M *NaCl* to a final concentration of 192 mM and incubate at 65°C for 4 h. Samples can be stored at -20°C at this time.
33. For DNA purification and subsequent polymerase chain reaction (PCR) analysis add 9 µl of 0.5 M *EDTA*, 18 µl 1 M *Tris HCl pH 6.5*, and 1.8 µl of a 10 mg/ml *proteinase K* solution to each sample.
34. Incubate for 1 h at 45°C.
35. Add 450 µl Roti-phenol/chloroform/isoamyl alcohol (25:24:1) and vortex each sample for 1 min (*see Note 21*).
36. Centrifuge the samples at 13,000 x g for 5 min at RT.
37. Transfer the supernatant to a fresh tube containing 450 µl chloroform/isoamyl alcohol (24:1) and repeat the extraction step (*see Note 21*).
38. Transfer the supernatant to a fresh 1.5-µl reaction tube, and add 50 µl of 3 M *sodium acetate pH 5.2*, 1 µl of 20 µg/µl glycogen as an inert carrier, 1 ml of absolute ethanol.
39. Mix and incubate at -80°C for 90 min.
40. Centrifuge the samples at 15,000 x g for 30 min.
41. Discard the supernatant carefully and wash the pellet with 1 ml of 70% (v/v) ethanol.
42. Repeat the centrifugation for 15 min, discard the supernatant, and dry the pellet in a Speed-Vac.
43. Resuspend the pellet in 50 µl of sterile H₂O and store at -20°C. Use 5-10 µl for the PCR (25-35 cycles) reaction. Appropriate PCR conditions have to be determined depending on the melting temperatures of primers and template.
44. Visualize PCR products by agarose gel electrophoresis and quantify ethidium bromide stained bands (*see Fig. 2*). Alternatively, PCR products can be quantified by real time PCR.

3.11 Analysis of *myc* expression by in situ hybridization

1. Cultivate the strains *Hydra vulgaris* or *Hydra magnipapillata* at 18°C and feed daily with freshly hatched *Artemia nauplii*. For usage in experiments, collect animals 24 h after the last feeding (12).
2. For whole mount in situ hybridization with digoxigenin-labeled RNA probes, proceed as described in (12, 16) (see Fig. 4).
3. For in situ hybridizations in single cell preparations, macerate *Hydra* as described in (17). Then fix macerates in paraformaldehyde and hybridize as described in (12, 18).

4 Notes

1. The first decantation of the cell suspension may be difficult because of high viscosity. If necessary, do not decant the entire supernatant in order to avoid transferring cell clumps. During the next extraction steps, the viscosity of the cell suspension is significantly lower.
2. Check complete detachment of trypsinized cells under the microscope.
3. Do not leave the viral stock longer at 37°C as necessary. Immediately after thawing add the virus to the cells. Also, avoid repeated freeze-thaw cycles of viral stocks. Ideally they are stored in aliquots and used only once.
4. After seeding, carefully move the dishes in horizontal and vertical directions to distribute the cells evenly on the surface. Do not swivel the dishes circularly! Repeat the rocking step after 30 min and after 60 min to avoid that most of the cells settle in the middle of the dish. That would result in an uneven cell distribution which is detrimental for foci scoring.
5. Use wide-mouth pipets for slowly dropping these solutions onto the cells. Distribute the single drops over the entire cell surface to minimize any stress caused by splashing liquid onto the cells which are very sensitive during this transfection step. After glycerol shock and washing, check cell viability under the microscope. Cells may look slightly different after the shocking procedure but they will recover soon.
6. Prepare serial dilutions using *transfer buffer* as a diluent.
7. Boiling of agar or agarose preparations in a microwave oven requires special care. Wear safety goggles! Check that the agar has been completely dissolved and put the hot glass vessel into the 45°C-water bath. Agar clumps in the overlay disturb the transparency and make scoring of foci or colonies difficult.
8. Pasteur pipets which have a ~ 30° kink approximately 1 cm away from the tip are favorable because foci are then easier to detach from the dish. To produce such a device, hold a pipet into the tip of a Bunsen burner flame and wait until the glass melts locally. Then, immediately remove the pipet from the flame, let it cool, and sterilize.

9. Use a flexible polyethylene tube attached with one end to the narrow site of a blue tip. Fill the tip with a cotton plug and use this inverted tip as a mouthpiece. The other end of the tube becomes attached to the kinked Pasteur pipet.
10. Let the agar harden for at least 2 h at RT not exceeding 25°C.
11. Change the tip when you enter the CsCl-phase. Try to remove any suspended precipitates of DNA or proteins before inverting the tube.
12. Pipet up and down several times to dissolve the RNA pellet completely.
13. Use a sterile rubber policeman to scrape the lysate from the dish. Afterwards, decontaminate the scraping device by submerging it in a 1% (w/v)-SDS solution for several days.
14. Take care that no material from the sepharose pellet becomes loaded onto the SDS-polyacrylamide gel.
15. Addition of specific antibodies directed against the DNA binding protein may either lead to a ternary antibody-protein-DNA complex with lower gel electrophoretic mobility, or to inhibition of DNA binding. Latter can be observed when the epitope of the antibody is localized in the DNA binding domain of the protein.
16. Remove the upper glass plate and leave the gel on the lower one. Submerge plate with attached gel in acetic acid solution. After fixing, use the upper glass plate to invert the gel.
17. Rock the dish immediately after addition of lysis buffer to quickly cover all cells.
18. Use aliquots of luciferase reagent stored at -80°C. Once the reagent is thawed, keep it protected from light.
19. The tip of the sonifier should be immersed as close as possible towards the bottom of the tube but not touch it. Otherwise foam could be generated by the sonication which makes subsequent pipetting difficult.
20. The optimal number of sonication cycles depends on the cellular material and can be determined in a pilot experiment with subsequent analysis of the chromatin fragment sizes obtained after various cycles of sonication.
21. It is important to remove the upper aqueous phases as accurate as possible for quantitative recovery of the released DNA fragments.

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Fig. 1. Transformation of quail embryo fibroblasts by the v-myc oncogene.

(A) *Top left panels:* phase contrast micrographs of normal quail embryo fibroblasts (QEF) and of the MC29-transformed quail cell line Q8 (2). *Bottom left panels:* colony formation by Q8 cells in soft agar, in comparison to control QEF. *Right panel:* immunoprecipitation and sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of radiolabeled v-Myc and endogenous c-Myc proteins from Q8 or QEF cell extracts, respectively. (B) *Left panels:* focus formation of QEF transfected with a plasmid construct containing the MC29 provirus (pRCAS-MC29), in comparison to control QEF. *Right panel:* map of pRCAS-MC29. Bright field micrographs were taken two weeks after DNA transfection. Modified and adapted from (13, 14).

Fig. 2. Transcriptional activation of the WS5 target gene by the v-Myc oncoprotein.

(A) Northern analysis using poly(A)⁺-selected RNAs from normal QEF, the v-myc (MC29) transformed cell line Q8, QEF transfected by pRCAS-MC29, and from quail cell lines transformed by the oncogenes v-src [R(-)3], v-jun (VJ), or chemically transformed by methylcholanthrene (QT6). Hybridization was performed using cDNA probes specific for *WS5*, v/c-myc, and the glycerol aldehyde phosphate dehydrogenase (*GAPDH*) gene. (B) Chromatin immunoprecipitation (ChIP) analysis of Q8 and QEF cells using polyclonal antisera directed against v/c-Myc, Gag, Max, or normal rabbit serum (NRS). Polymerase

chain reaction (PCR) analysis was performed to amplify a *WS5* promoter specific region containing a cluster of four Myc binding sites (E-boxes). Modified and adapted from (15).

Fig. 3. Biochemical properties of recombinant *Hydra* Myc and Max proteins.

(A) SDS/PAGE of 2- μ g (Coomassie Brilliant Blue staining) or 50-ng (immunoblotting) aliquots of purified recombinant *Hydra* Myc1 p16, *Hydra* Max p22, and a 1:1 mixture of both proteins. (B) EMSA using the recombinant proteins shown in A and a radiolabeled double-stranded deoxyoligonucleotide containing the Myc/Max-binding motif 5'-CACGTG-3'. Antibodies were added to the binding reactions as indicated. Modified and adapted from (12).

Fig. 4. Expression of *Hydra myc1* and *max*.

(A, B) In situ hybridization showing that *myc1* and *max* are activated in cells belonging to the interstitial stem cell lineage in the gastric region of intact, budding polyps. In addition, *max* is expressed at a lower level in the epithelium throughout the entire body column. (C) Northern analysis using poly(A)⁺-selected RNAs from whole *Hydra* animals and *myc1* or *max* specific cDNA probes. Modified and adapted from (12).