Index

A

Adenoviral gene transfer, see Cyclic AMP; High-throughput screening, phosphodiesterase activity in living cells; PDE3B

B

Baculovirus-Sf9 cell expression system, cygnet probes 31, 38–40
PDE6αβ/PDE5 chimera expression in Bac-to-Bac system 266
Blastocyst, knockout mouse generation, microinjection of embryonic stem cells 204
preparation for microinjection 203, 204, 207
transfer 205

C

Calcium/calmodulin-stimulated phosphodiesterases, see PDE1
Calcium flux, fluorescence measurements for phosphodiesterase activity screening,
Fura-2 loading 49, 51, 52
PDE4 activity assay in vascular smooth muscle cells 54
ratio fluorescence measurements in cell suspensions 49
single-cell assay 52
cAMP, see Cyclic AMP
cGMP, see Cyclic GMP
Crystallization, phosphodiesterases, factors affecting 183
materials 182, 188
overview 181, 182
PDE4 crystallization, microdialysis 187–188
vapor diffusion 186, 187
PDE5 crystallization by vapor diffusion 186, 187, 188, 189
practical aspects 185, 186, 188–189
stages 182, 183
techniques,
batch crystallization 183
microdialysis 184
seeding 184, 185
vapor diffusion 183, 184
Cyclic AMP (cAMP), compartmentation 16
effectors 15
fluorescence resonance energy transfer imaging in living cells, calculations 4, 11
cell culture and transfection 8, 12
image acquisition 8, 9, 12
image processing and analysis 9
instrument setup 5–7, 12
materials 4, 5, 12
principles 2–4
protein kinase A probes 2–4
spectral overlap between donor and acceptor 3, 9
olfactory cyclic nucleotide-gated channels for measurement in single cells,
calibration 19, 21
cell culture and adenoviral transfection 17, 23
compartmental models of localized signals 21–25
materials 16, 17, 23, 24
overview 15, 16, 23
patch-clamp measurements, overview of technique 18
perforated patch experiments 18, 19, 23, 24
whole-cell experiments 18, 24
phosphodiesterase activity screening using cyclic nucleotide-gated channels, see High-throughput screening, phosphodiesterase activity in living cells
radioimmunoassay 1
Cyclic GMP (cGMP), binding studies, see PDE5; PDE11 effectors 75
fluorescence resonance energy transfer imaging in single cells, aortic smooth muscle cell culture 37
cygent probes, baculovirus-Sf9 cell expression system 31, 38–40
cloning 30, 31
design 28, 30
fluorescence spectroscopy 34, 41
kinase activity 34
plasmids 29, 30
purification 31–33, 40
titration and dissociation constant determination 34, 35
image acquisition 38, 41
materials 28, 29
principles 28
RFL-6 fibroblast culture 35, 37
transfection 37, 38, 41
GAF regulatory domain binding in phosphodiesterases, see PDE6
Cyclic nucleotide-gated channels, see Cyclic AMP; High-throughput screening, phosphodiesterase activity in living cells
Cygnets, see Cyclic GMP

D
Dimerization, see Subunit structure, phosphodiesterases

E
Electrophoretic mobility shift assay (EMSA), PDE3B promoter elements, electrophoresis 117
nuclear extract preparation 115
probe preparation 116
EMSA, see Electrophoretic mobility shift assay
ERK2, see Extracellular signal-regulated kinase 2
Extracellular signal-regulated kinase 2 (ERK2), PDE4 phosphorylation studies, immunoprecipitation of PDE4 230, 235
materials 226, 227
overview 226
phosphorylation in vitro, incubation conditions 231, 232, 236
maltose binding protein-PDE4D3 fusion protein purification 230, 231
phosphorylation in vivo, immunoprecipitation 234, 235
radiolabeling of immunoprecipitates 235
radiolabeling using orthophosphate 232–234
transfection of Cos-1 cells and phosphodiesterase assay 227–229

F
Fluorescence resonance energy transfer (FRET), cyclic AMP imaging in living cells, calculations 4, 11
cell culture and transfection 8, 12
image acquisition 8, 9, 12
image processing and analysis 9
instrument setup 5–7, 12
materials 4, 5, 12
Index

principles 2–4
protein kinase A probes 2–4
spectral overlap between donor and acceptor 4, 9
cyclic GMP imaging in single cells with cygnets,
aortic smooth muscle cell culture 37
cygnet probes,
baculovirus-Sf9 cell expression system 31, 38–40
cloning 30, 31
design 28, 30
fluorescence spectroscopy 34, 41
kinase activity 34
plasmids 29, 30
purification 31–33, 40
titration and dissociation constant determination 34, 35
image acquisition 38, 41
materials 28, 29
principles 28
RFL-6 fibroblast culture 35, 37
transfection 37, 38, 41

FRET, see Fluorescence resonance energy transfer
Fura-2, see Calcium flux

G
GAF regulatory domain, see PDE6
GARP2, see Glutamic acid-rich protein-2

Gel filtration,
PDE4 subunit structure studies, chromatography 172, 175
materials 170, 171
molecular weight calculation 174–176
principles 168–170
sample preparation 171, 172, 175
PDE6 dimerization determinant analysis 270, 271
Glutamic acid-rich protein-2 (GARP2), PDE6 binding and removal from preparations 132, 133, 138

H
High-throughput screening, phosphodiesterase activity in living cells,
cyclic nucleotide-gated channels as cyclic AMP probes,
calcium flux measurements in HEK-293 cells,
Fura-2 loading 49, 51, 52
ratio fluorescence measurements in cell suspensions 49
single-cell assay 52
cell culture and expression 47, 57
inhibition constant estimation for inhibitors in living cells 54, 55, 58
PDE type IV activity assay in vascular smooth muscle cells 54
rat olfactory CNGA2 channel electrophysiological characteristics expressed in HEK-293 cells 50
sensitivity testing with patch-clamp 47–49
materials 46, 47, 57
overview 45, 46

I
Immunoprecipitation, see PDE2; PDE4; PDE6
Inclusion body, advantages in recombinant protein expression 156, 157
avoidance of accumulation 156
phosphodiesterase renaturation, see PDE4A; PDE7A
Index

In situ hybridization, PDE9 localization
in rat brain,
hybridization 80
immunostaining with neuronal and
glial markers for cell type
identification 82, 83
materials 76, 77
overview 75, 76
pretreatment of tissue sections 79, 80
riboprobe preparation,
concentration determination 79
design 78
digoxigenin labeling 78, 79
RNase contamination prevention 77, 78
tissue section preparation 79
washing and color development 80, 82, 83

K
Knockout mice, see PDE4

M
Microdialysis, see Crystallization,
phosphodiesterases
Mitogen-activated protein kinase, see
Extracellular signal-regulated kinase 2

N
Nerve growth factor (NGF), mediation
of PDE2 inactivation 211, 212
NGF, see Nerve growth factor

P
Patch-clamp, cyclic AMP
measurements,
phosphodiesterase activity screening
using cyclic nucleotide-gated channels,
rat olfactory CNGA2 channel
electrophysiological characteristics expressed in
HEK-293 cells 50
sensitivity testing 47–49
single cells using olfactory cyclic
nucleotide-gated channels,
calibration 19, 21
cell culture and adenoviral
transfection 17, 23
compartmental models of
localized signals 21–25
materials 16, 17, 23, 24
overview 15, 16, 23
patch-clamp technique overview 18
perforated patch experiments 18, 19, 23, 24
whole-cell experiments 18, 24
PCR, see Polymerase chain reaction
PDE, see Phosphodiesterase
PDE1,
activation assay,
angiotensin II effects on
activation 90, 91
cell lysis 87, 90
chromatography 88–90
homogenization of tissue 87, 88
incubation conditions 88, 90
materials 86, 87
principles 86, 88, 89
scintillation counting and
calculations 90
snake venom treatment of samples 88
calcium activation and sensitivity 85, 86
genes in family 85
splice variants 85
PDE2,
activity assays 216, 220, 221
coimmunoprecipitation of PDE2A2
with associated protein kinase,
antibody production 214, 215, 220
FLAG-tagged PDE2A2
construction and expression 213, 214, 220
homogenization and solubilization 215, 220, 221
immunoprecipitation 215, 221
materials 212, 213
phosphorylation in vitro 215, 216
nerve growth factor-mediated
inactivation 211, 212
tissue distribution 211

PDE3B,
adeno viral transfer of mouse gene,
adeno virus quantification,
particle number 99
plaque assay 100, 102
real-time polymerase chain
reaction 99, 100, 106
cotransfection of transfer plasmid
and pJM17 in HEK-293 cells
95, 96
infection and expression,
applications 104, 106
FDCP2 cells 103, 104, 106
H4IIE hepatoma cells 102
pancreatic cells 104
large-scale virus preparation,
cell harvesting 97, 98
cesium chloride gradient
centrifugation 98
dialysis 98
infection of HEK-293 cells 96,
97, 106
materials 94, 95
overview 94
subcloning into adenovirus
transfer plasmid 95
promoter element identification in 5’
flanking region of mouse gene,
cloning of flanking region 111,
123
electrophoretic mobility shift
assay,
incubation conditions and
electrophoresis 117
nuclear extract preparation 115
probe preparation 116
materials 110, 111
overview 109, 110

reporter plasmids,
construction 111–113
luciferase assay 115
transient transfection 113–115
translation initiation site mapping,
messenger RNA isolation 117,
119
rapid amplification of cDNA
ends 119, 121–123
ribonuclease protection assay
119, 121, 123
tissue distribution 109, 110

PDE4,
activity screening in vascular smooth
muscle cells 54
crystallization,
microdialysis 187–188
vapor diffusion 186, 187
extracellular signal-regulated kinase
2 phosphorylation studies,
immunoprecipitation of PDE4
230, 235
materials 226, 227
overview 226
phosphorylation in vitro,
incubation conditions 231, 232,
235
maltose binding protein-
PDE4D3 fusion protein
purification 230, 229
phosphorylation in vivo,
immunoprecipitation 234, 235
radiolabeling of
immunoprecipitates 235
radiolabeling using
orthophosphate 232–234
transfection of Cos-1 cells and
phosphodiesterase assay 227–
230
functions 226
genes 191
inhibitors 191, 192
knockout mice generation,
chimera preparation,
blastocyst preparation for microinjection 203, 204, 207
blastocyst transfer 205
embryonic stem cell preparation for microinjection 203
microinjection of embryonic stem cells into blastocysts 204
mouse strains 203, 207
pseudopregnant female preparation 204, 205, 207
colony picking and expansion following electroporation 199, 200
embryonic stem cell clone expansion and freezing 201, 202
embryonic stem cell clone screening for homologous recombinants,
DNA extraction 200, 201
overview 194, 195, 206, 207
Southern blot 201
embryonic stem cell transfection, electroporation 199, 207
feeder cell preparation 198, 199
genotyping 206–208
karyotyping of embryonic stem cells 202, 203
materials 195–197
mating of chimeras 205, 206
PDE4B gene targeting vector 197, 198, 207
rationale 192
strategies 192–194
renaturation of recombinant PDE4A catalytic domain expressed as inclusion bodies,
expression in Escherichia coli 158, 159, 162
inclusion body purification 159, 162
materials 158, 162
purification of refolded enzyme 159, 160
refolding condition optimization 160–163
solubilization and refolding 159, 162, 163
structure 226
subunit structure,
dimerization domain mapping 168
domains 168
full-length protein study limitations 168
gel filtration studies,
chromatography 172, 177
materials 170, 171, 175
molecular weight calculation 174, 176
principles 168–170
sample preparation 171, 172, 175
sucrose density gradient centrifugation studies,
centrifugation 172–174, 177, 178
materials 170, 171, 177
molecular weight calculation 174, 176
principles 168–170, 175
sample preparation 171, 172, 175
tissue distribution 191
PDE5,
 crystallization by vapor diffusion 186, 187
cyclic GMP/analog binding studies,
cyclic GMP binding to allosteric sites,
binding specificity 246, 247
inhibitor and divalent cation effect studies 247, 260
overview 245
pH, protein, and ionic strength effects 249–251, 253, 255
protein concentration and preparation 246
reagents 245, 246
temperature effects 246
materials 243
multisample filter assay,
advantages and limitations 257–260
principles 243, 244
sildenafil binding 254, 255, 260
overview 240, 243, 244
radioligand study advantages 241–243
sildenafil binding assays,
catalytic site binding assay 257, 258
equilibration time 255
exogenous protein, EDTA, and detergent effects 256, 257
ionic strength effects 256
temperature effects 256
single-well filter assay,
advantages and limitations 258–260
principles 243, 244
sildenafil binding 253
technique 244, 245, 260
PDE6 chimeras, see PDE6
structure 240
PDE6,
cyclic GMP binding assays for PDE6R GAF regulatory domain,
centrifugal separation of membrane-associated enzyme through silicone oil,
centrifugal tube preparation 148, 151
centrifugation and quantification of cyclic GMP 149, 152
principles 148
rod outer segment preparation and incubation 149, 152
validation 149, 152
interpretation 149, 150
materials 142, 143
membrane filtration assay, ammonium sulfate stabilization of binding 146–148, 151
dissociation assessment during membrane filtration 146
enzyme recovery assessment on filter 145
incubation conditions 144, 145, 151
nonspecific binding determination 145, 146
overview 141, 142
PDE6R preparation, endogenous cyclic GMP removal 143, 144
enzyme inhibition to prevent ligand destruction 144, 151
purification 143
function 125, 277
isoforms 125, 126
Pγ inhibitory subunit, displacement by transducin 277
mutant studies of catalytic subunit interactions,
activity assay for inhibition testing 284
cloning 280, 284
fluorescence binding assay 283, 285, 286
fluorescent probe labeling 282, 283, 285
materials 278, 279
overview 277, 278
pET system for expression 279, 281, 282
photocrosslinking 283, 284, 286
polymerase chain reaction-directed mutagenesis 280, 281, 284, 285
purification 282, 283
removal from enzyme preparations, proteolytic fragment removal 137–139
trypsinization 137
structure 277
posttranslational modification 289, 290
puriﬁcation from mammalian retina, concentration with ultraﬁltration 136, 137
homogenate preparation 129, 138
materials 126, 127
overview 127, 128
PDE6C,
hydroxyapatite chromatography 135, 139
imunoaﬃnity chromatography 135, 136, 139
separation from soluble PDE6R with anion-exchange chromatography 133, 134, 139
PDE6R,
anion-exchange chromatography 131, 132
binding protein removal with hydrophobic interaction chromatography 132, 133, 138
extraction from rod outer segments 130, 131
gel ﬁltration 133, 139
rod outer segments,
purification 129, 130, 138
separation from soluble retinal proteins 129
soluble PDE6 extraction 129
reconstitution on lipid vesicles,
activity assay 309, 310
extraction from rod outer segments 296–299
lipid vesicles,
binding assays 308, 309
preparation 305–308, 311, 312
materials,
apparatus 295, 296
assay reagents 294, 295
biological materials 290, 291
buffers 291–293
chromatography and ﬁltration media 293, 294
PDE6 puriﬁcation,
anion-exchange chromatography 301
gel ﬁltration 301, 302
hydroxyapatite chromatography 300, 301
overview 299, 300
transducin,
puriﬁcation with hexylagarose chromatography 302, 303
subunit separation with Blue Sepharose chromatography 303–305
subunit structure 263, 264
transducin activation 289, 290
PDE7A, refolding from inclusion bodies 160–163
PDE9, in situ hybridization for localization in rat brain,
hybridization 80
immunostaining with neuronal and glial markers for cell type identification 82, 83
materials 76, 77
overview 75, 76
pretreatment of tissue sections 79, 80
riboprobe preparation,
concentration determination 79
design 78
digoxigenin labeling 78, 79
RNase contamination prevention 77, 78
tissue section preparation 79
washing and color development 80, 82, 83
PDE11, cyclic GMP/analog binding studies,
materials 243
multisample ﬁlter assay,
advantages and limitations 257–260
principles 243, 244
overview 240, 243, 244
Index 323

radioligand study advantages 241–243
single-well filter assay,
advantages and limitations 258–260
principles 243, 244
technique 244, 245, 260
Pγ, see PDE6
Phosphodiesterase (PDE), see also
specific PDEs,
calcium/calmodulin-stimulated phosphodiesterases, see PDE1
classification 45, 46, 63–65, 75, 94, 167, 168
crystallization, see Crystallization, phosphodiesterases
function 63, 93
high-throughput screening, see High-throughput screening, phosphodiesterase activity in living cells
kinases, see Extracellular signal-regulated kinase 2; PDE2
knockout mice, see PDE4
radioenzymatic assay with inhibitors for isozyme determination,
calculations 69, 70
enzymatic reactions 68, 69, 71
homogenization of tissue 66, 71
interpretation 70–73
materials 64, 66
principles 66
QAE-Sephadex A-25 formate anion-exchange resin,
chromatography 69
preparation and equilibration 68
regeneration 68
radiolabeled cyclic nucleotide purification 66, 67, 71
subunit structure, see Subunit structure phosphodiesterases,
PKA, see Protein kinase A
PKG, see Protein kinase G
Polymerase chain reaction (PCR),
adenovirus quantification with real-time polymerase chain reaction 99, 100, 106
PDE4 knockout mouse genotyping 206–208
PDE6αβ/PDE5 chimera cloning 269, 270
Pγ inhibitory subunit mutagenesis 280, 281, 284, 285
Protein kinase A (PKA), probes for cyclic AMP fluorescence resonance energy transfer imaging in living cells 2–4
Protein kinase G (PKG), cygnet probes for cyclic GMP imaging, see Cyclic GMP
R
RACE, see Rapid amplification of cDNA ends
Rapid amplification of cDNA ends (RACE), translation initiation site mapping in mouse PDE3B 119, 121–123
Reconstitution, see PDE6
Ribonuclease protection assay (RPA),
translation initiation site mapping in mouse PDE3B 119, 121
RPA, see Ribonuclease protection assay
S
Sildenafil (Viagra),
radioligand binding studies to PDE5
advantages 241–243
assay parameters,
catalytic site binding assay 257, 258
equilibration time 255
exogenous protein, EDTA, and detergent effects 256, 257
ionic strength effects 256
temperature effects 256
multisample filter assay, advantages and limitations 257–260
principles 243, 244
sildenafil binding 254, 255, 260
overview 240, 243, 244
single-well filter assay, advantages and limitations 258–260
principles 243, 244
sildenafil binding 253
technique 244, 245, 260
structural homology with cyclic GMP 242
Southern blot, PDE4 knockout mouse genotyping 201, 206–208
Subunit structure, phosphodiesterases, dimerization domain mapping 168
domains 168
full-length protein study limitations 168
gel filtration studies, chromatography 172, 177
materials 170, 171, 175
molecular weight calculation 174, 176
principles 168–170
sample preparation 171, 172, 175
PDE6 dimerization determinant analysis, dimerization assays, gel filtration 270, 271
immunoprecipitation 271, 272
sucrose density gradient centrifugation 272, 273
Western blot 271
materials 264, 265
overview 263, 264
PDE6αβ/PDE5 chimeras, Bac-to-Bac expression system 266
cloning approaches 266, 267, 269, 272
expression in Sf9 cells 270, 272
polymerase chain reaction-based cloning 269, 270
sucrose density gradient centrifugation studies, centrifugation 172, 174, 177, 178
materials 170, 171, 175
molecular weight calculation 174, 176
principles 168–170
sample preparation 171, 172, 175
Sucrose density gradient centrifugation, PDE4 subunit structure studies, centrifugation 172–174, 177, 178
materials 170, 171, 177
molecular weight calculation 174, 176
principles 168–170
sample preparation 171, 172, 175
PDE6 dimerization determinant analysis 272, 273
T
Tadalafil, structural homology with cyclic GMP 242
Transducin, see PDE6
Translation initiation site mapping, see PDE3B
V
Vapor diffusion, see Crystallization, phosphodiesterases, Viagra, see Sildenafil
W
Western blot, PDE4 230
PDE6 dimerization determinant analysis 271, 272
X
X-ray crystallography, see Crystallization, phosphodiesterases