Comprehensive Testing for *NF1/SPRED1* and Other RASopathies Genes at UAB
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a comprehensive menu allowing a tailored approach for patients with constitutional or mosaic presentations

| DNA-based testing by NGS (effective April 18th, 2016) | • NF1-only
• NF1-SPRED1
• Expanded NF1-RASopathy (16 genes)
• non-NF1 RASopathy (15 genes) | NF1, SPRED1, PTPN11, BRAF, CBL, HRAS, KRAS, NRAS, MAP2K1, MAP2K2, RAF1, RIT1, RASA2, SHOC2, SOS1 and SOS2 (16 genes) |
| RNA-based testing by Sanger | Comprehensive NF1/SPRED1 testing on blood and affected tissues |

BACKGROUND

The RASopathies are a genetically heterogeneous group of disorders caused by mutations in the genes involved in the Ras-MAPK pathway. As a group, the RASopathies are one of the largest groups of malformation syndromes known, affecting ~1:1,000 and include Neurofibromatosis type 1, Legius syndrome, Noonan syndrome, cardio-facio-cutaneous (CFC) syndrome, Noonan Syndrome with Multiple Lentigines (NSML/-LEOPARD) and Costello syndrome. Mutations in NF1 and SPRED1 are typically loss-of-function mutations and include the full spectrum of nonsense, missense, splice, frameshift, insertion-deletion, and copy number changes. Mutations in the other RASopathy genes are typically missense mutations or/in-frame deletion/insertion of an amino acid.

The Ras/MAPK pathway can have a profound deleterious effect on development as it plays a key role in differentiation, growth, senescence, and dysregulation. Clinical features of the RASopathies include short stature; cardiovascular defects; cutaneous and pigmentary findings; characteristic faces; skeletal and neuro-cognitive delays as well as a predisposition to neoplasia, both benign and malignant. The disorders have variable expressivity (individuals with the same disorder may show differing features and severity of symptoms, even within the same family). Some of the genes/mutations are not fully penetrant, therefore an individual may carry a mutation but not show any or only few signs of the syndrome. Moreover, features can change/progress with age, which makes it difficult to make an accurate clinical diagnosis. The RASopathies are inherited in an autosomal dominant manner. A parent who carries a mutated gene has a 50% chance of passing it on to every child, regardless of gender. An individual can carry a mutation either a) because (s)he inherited the mutation from a parent (parent clinically affected or "non-penetrant"), or b) because the mutation arose “de novo” in the egg or sperm from which the individual developed. Sometimes, the mutation occurred “post-zygotically”, i.e. during development and in these individuals the mutation may not be present in every cell of the body, typically resulting in a more mild phenotype (mosaicism).
Neurofibromatosis type 1
The NF1 gene, cloned in 1990, was the first gene within the Ras-MAPK pathway shown to be associated with an autosomal dominant disorder, Neurofibromatosis type I (NF1). NF1, affects ~1/3000 individuals worldwide, with half of the patients being sporadic. NF1 is notorious for its phenotypic variability and is a progressive disorder with more signs developing with time. Although the NIH criteria enables clinicians to make a diagnosis in the majority of classically affected cases, diagnostic criteria are not met until a given age is reached. Atypical presentations also exist with patients not yet fulfilling NIH criteria by adulthood. The mutational spectrum of NF1 is very complex and includes a wealth of unusual splice mutations affecting exonic sequences as well as deep intronic mutations resulting in exonization of intronic sequences at the mRNA level.

Legius syndrome
Germline loss-of-function mutations in SPRED1, a negative regulator of the RAS-MAPK pathway, cause a neurofibromatosis type 1-like phenotype, first described in 2007 (Legius syndrome). Patients present with multiple café-au-lait spots with or without skinfold freckling. Other typical NF1 associated features (Lisch nodules, bone abnormalities, neurofibromas, optic pathway gliomas) are systematically absent. However, in some individuals Noonan-like features have been reported.

Noonan syndrome
Noonan syndrome (NS), Noonan Syndrome with Multiple Lentigines (NSML, aka LEOPARD) and Noonan syndrome with “loose anagen hair” are autosomal dominant disorders affecting ~1:1,000-2,000 individuals. Patients present with craniofacial features and a variable clinical phenotype including congenital heart defects, reduced growth, bleeding disorders (NS), and variable degrees of neurocognitive delay. Patients with NSML also have multiple lentigines, genital abnormalities and sensorineural deafness. Patients with NS also have an increased cancer predisposition. Genes associated with NS and NSML are PTPN11, KRAS, SOS1, RAF1, NRAS, Braf, MAP2K1, CBL, RIT1, RASA2 and SOS2. The SHOC2 gene is associated with NS with “loose anagen hair” or sparse slow growing hair.

Cardio-Facio-Cutaneous syndrome
Cardio-Facio-Cutaneous syndrome (CFC) is a rare condition with genetic and phenotypic overlap with NS. Clinical features include craniofacial features similar to those found in NS, neurocognitive delay, failure to thrive, congenital heart defects, epilepsy and a wide range of ectodermal manifestations. Four genes have been associated with CFC: BRAF, MAP2K1, MAP2K2 and KRAS.

Costello syndrome
Costello syndrome (CS), caused by activating HRAS mutations, is a very rare condition with the following key features: coarse facial features, severe feeding difficulty, mild to moderate intellectual disability, relative macrocephaly and short stature, high incidence of cardiac abnormalities and malignancy. Differentiation of CS from other RASopathies, particularly CFC may be difficult especially early in life.
Some individuals with a clinical diagnosis of one of the RASopathies have been found to carry a mutation in a gene that was not considered to be consistent with their clinical diagnosis. Examples include *BRAF* variants reported in individuals with a clinical diagnosis of Noonan syndrome, a *SOS1* variant in an individual with CFC (Nystrom AM et al, 2008), *PTPN11* mutations in individuals with paraspinal neurofibromas (Conboy E. et al, 2015), and an *NF1* missense mutation in patients with Noonan-like features and no neurofibromas (Rojnueangnit K et al, 2015). In addition, some genes are associated with more than one syndrome (*PTPN11, KRAS, BRAF, RAF1, NF1*). Therefore, the comprehensive approach of simultaneously testing all 16 genes in some individuals eliminates the need to determine which genes to test based on an individual’s clinical signs.

For further information, the review articles references on page 8 can be consulted.
**The average coverage is 1,800X** with >99.5% of the NF1 regions >200X and <0.01% at <100X but still >50X, allowing detection of very low level mosaicism by sequencing (down to 8% of the alleles and lower). Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels, and frameshifts caused by deletion/duplication up to 64bp.

Based on >15 years of experience with comprehensive RNA-based NF1 testing, we designed the **customized and optimized NGS NF1-component** of the assay to comprise all regions encountered through analysis of >15,000 unrelated individuals including >8,100 NF1-mutation-positive individuals carrying 1 out of >3,100 different unique NF1 mutations identified in the UAB MGL cohort. Included in the NGS assay are the regions covering >65 different deep intronic splice mutations (which reside beyond the +/-50 intronic base pairs that flank all exons). Validation of the full panel included, besides substitutions (missense, nonsense, splice variants), the most challenging mutations such as insertions/deletions/duplications of 1-64 bp (~25% of the UAB NF1 cohort) and one-to-multiple exon deletions/duplications (~2.8% of the UAB NF1 cohort). The analytical sensitivity of our NGS testing approach was 100% for substitutions as well as insertion/deletions up to 64bp. This panel has not yet been validated to identify deletions/duplications >64bp and <1 exon, as such mutations have not yet been found in the UAB cohort, and therefore are likely very rare. The panel has been validated for the detection of germine (heterozygous) single-exon deletions/duplications, however mosaic single exon deletion/duplications validation is still pending. Single exon deletions/duplications are present in ~0.45% of NF1-positive patients from the UAB cohort with 9% of these individuals being mosaic (~0.045% of all in the UAB NF1-positive cohort). Detection of Alu/LINE insertions, identified in 0.25% of patients from the UAB NF1-positive cohort, has not yet been validated using the current NGS approach.

With the **largest dataset of NF1 genotypes matched with phenotypes**, any genotype-phenotype correlations identified will be reported in real time.

**Confirmatory testing of reportable variants** is performed using orthogonal methods as needed.

For **novel NF1 variants of unknown significance**, we offer free of charge targeted RNA-based testing to assess the effect of the variant on splicing and enhance the correct classification/interpretation. Relevant family members of a proband with any (novel or previously identified) variant of unknown significance are offered free of charge targeted analysis as long as accurate phenotypic data are provided by a health care professional to enhance the interpretation. There is no limitation to the number of relatives that can be tested free of charge.

**Mosaicism** is often present in sporadic patients with an NF1 microdeletion and has important repercussions for counseling. Free of charge evaluation by **FISH analysis on 200 interphase chromosomes** is offered in such cases.
**NF1/SPRED1-only by NGS**

**Candidates for this test:** patients with multiple CALMs w/wo skinfold freckling and no other typical NF1 associated features (Lisch nodules, bone abnormalities, neurofibromas, optic pathway gliomas).

The DNA-based **NF1/SPRED1-only by NGS** involves **sequencing** as well as **deletion/duplication analysis** of the entire coding **NF1/SPRED1** regions plus the alternatively spliced **NF1** exons 9br, 23a and 48a (67 exons total). The test uses the same approach as detailed above (see: **NF1-only by NGS**). The **average coverage is 1,800X with >99.5% of the NF1 regions >200X** and <0.01% at <100X but still >50X, allowing detection of very low level mosaicism by sequencing (down to 8% of the alleles and lower).

**Expanded NF1-RASopathy by NGS**

**Candidates for this test:** patients with clinical features suggestive of either NS, NSML, CFC, NF1, Legius syndrome or Noonan-like syndrome; patients with a clinical diagnosis of any of these syndromes that previously tested negative in a subset of the genes included in this panel; patients with a diagnosis of Costello syndrome but no **HRAS** mutation previously identified.

The **Expanded NF1-Rasopathy panel by NGS** involves the simultaneous sequencing of **16 genes**: **NF1, SPRED1, PTPN11, BRAF, CBL, HRAS, KRAS, NRAS, MAP2K1, MAP2K2, RAF1, RIT1, RASA2, SHOC2, SOS1 and SOS2** (282 exons). The test uses the same approach as detailed above (see: **NF1-only by NGS**). The **average coverage is 1,800X with >99.5% of the NF1 regions >200X** and <0.01% at <100X but still >50X, allowing detection of very low level mosaicism by sequencing (down to 8% of the alleles and lower). Variant and copy number calls are made using a unique bioinformatics pipeline detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 64bp. Deletion/duplication analysis for **NF1/SPRED1** is included in this test, as such mutations are a part of the mutation spectrum for these conditions. Deletion/duplication analysis for the other 14 genes on this panel is not offered as current empirical and biological evidence is not sufficient to allow the conclusion that an altered copy number of these genes is a mechanism critical for the phenotype associated with the Rasopathies.

**Non-NF1 RASopathy by NGS**

**Candidates for this test:** patients with clinical features suggestive of either NS, NSML, CFC, Legius syndrome or Noonan-like syndrome with no mutation previously found by comprehensive RNA-based **NF1+/- SPRED1** testing.

The **non-NF1 Rasopathy by NGS** involves the simultaneous sequencing of 15 genes: **SPRED1, PTPN11, BRAF, CBL, HRAS, KRAS, NRAS, MAP2K1, MAP2K2, RAF1, RIT1, RASA2, SHOC2, SOS1 and SOS2** (220 exons). The test uses the same approach as detailed above (see: **NF1-only by NGS**). The **average coverage is 1,800X with >99.5% of the NF1 regions >200X** and <0.01% at <100X but still >50X, allowing detection of very low level mosaicism by sequencing (down to 8% of the alleles and lower). Variant and copy number calls are made using a unique bioinformatics pipeline
detecting all types of mutations including single nucleotide substitutions, indels and frameshifts caused by deletion or duplication up to 64bp. Deletion/duplication analysis for SPRED1 is included in this test, as such mutations are a part of the mutation spectrum for this gene. Deletion/duplication analysis for the other 14 genes on this panel is not offered as current empirical and biological evidence is not sufficient to allow the conclusion that an altered copy number of these genes is a mechanism critical for the phenotype associated

**RNA-based Testing**

**TEST OPTIONS**
- RUSH RNA-based NF1 and DNA-based SPRED1 testing on blood
- RNA-based NF1 testing on blood
- RNA-based NF1/SPRED1 testing on affected tissues

**SPECIMEN TYPES**
- Fresh EDTA blood sample, to arrive in the lab <60-70 hours after blood draw
- Biopsies of café-au-lait macules (CALM) and/or neurofibromas

**RUSH RNA-based NF1 and DNA-based SPRED1 testing**

**Candidates for this test:** patients who need the most sensitive and specific test with the fastest turnaround time.

The RUSH RNA-based NF1 and DNA-based SPRED1 testing on blood requires a fresh EDTA blood sample, to arrive in the lab <60-70 hours after blood draw. DNA is extracted and in addition, a short term phytohemagglutinin-stimulated lymphocyte culture is initiated and used as starting material to extract RNA. The complete NF1 coding region is analyzed by a cascade of complementary mutation detection techniques, including RT-PCR, direct sequencing of cDNA fragments, microsatellite marker analysis, copy number analysis by MLPA and interphase FISH (if needed), enabling identification of the mutation in ~95% of non-founder patients fulfilling the NIH diagnostic criteria (Messiaen et al, 2000; Messiaen and Wimmer, 2008).

**RNA-based NF1 testing** allows finding deep intronic splice mutations through their observed effect on splicing. These splice mutations would not be detected if a “simple” exon-by-exon DNA-based (NGS/Sanger) sequencing approach is used. During the >15 years we have offered comprehensive RNA-based NF1 testing on blood, we have identified >65 different deep intronic splice mutations: together they account for 2.5% of all pathogenic mutations identified in the NF1 UAB cohort. Please note that all known deep intronic splice mutations have been incorporated in the customized UAB NGS assay described above.

In addition, all coding exons and flanking intronic sequences of the SPRED1 gene are analysed by bidirectional sequencing and deletion/duplication analysis is performed using MLPA. Turnaround time for this test is 15 working days.
RNA-based NF1 testing

**Candidates for this test:** non-founder patients with a) clearcut classic NF1; b) from a clinically documented multi-generation (minimum 3 generations) family; c) who tested negative by the MGL NF1-only by NGS assay and d) in whom a translocation has been excluded by cytogenetic analysis. The latter patients will receive free of charge reflex RNA-based NF1 testing, which should allow to detect a possible deep intronic splice mutation not previously identified in the UAB cohort and not reported elsewhere, or a possible Alu/LINE insertion or other exotic complex mutation. Based on current data, such mutations account conservatively for <0.25% of familial cases.

NF1 testing is performed as described in the previous section.

RNA-based NF1/SPRED1 testing on cultured cells from affected tissues

**Candidates for this test:** Individuals suspected to have segmental NF1, with symptoms restricted to a defined area of the body; sporadic patients who have (mild) non-localized symptoms of NF1 but in whom no NF1 mutation was identified in the blood lymphocytes and may have disease due to a postzygotic mutation; reflex testing for familial or sporadic patients with a first hit mutation refractory to detection by RNA- or DNA/NGS assay.

The RNA-based NF1/SPRED1 testing on cultured cells from affected tissues is offered starting from biopsies of café-au-lait macules (CALM) and/or neurofibromas. Melanocytes cultured from CALMs and Schwann cells cultured from neurofibromas are the starting material for the cascade of complementary mutation detection techniques described in the previous section on “Comprehensive RNA-based NF1 testing on blood” (Maertens et al, 2007, De Schepper et al, 2007). In addition, for patients with only pigmentedary features (CALMs w/wo skinfold freckling but no neurofibromas), and no NF1 mutations found in the melanocytes (no first or second hit mutations), the SPRED1 gene is analysed at no additional charge (sequencing and deletion/duplication analysis), as these patients may have mosaic Legius syndrome. As a result of this test, if features are NF1 or SPRED1-related, a common first hit is identified in both biopsies and a different second hit is identified in every anatomically different biopsy evaluated. If no mutations are identified despite full analysis on 2 biopsies with successful cultures, (mosaic) NF1/Legius syndrome is very unlikely (<0.1%).

We require a minimum of 2 biopsies (2-3 mm punch biopsies) from anatomically different locations. Please contact us at medgenomics@uabmc.edu or 205-934-5562 to set up a time to discuss your patient prior to taking biopsy/biopsies in your patient, so we can provide individualized advice and ship out appropriate collection/transport media and forms prior to the procedure.

Please contact us if you have questions or want to discuss genetic testing needs for your patients. We will be happy to help.
References


The Medical Genomics Laboratory in the Department of Genetics at UAB, directed by Ludwine Messiaen, Ph.D., FACMG, is an academic non-for-profit clinical lab, CLIA and CAP certified. Our dedicated and experienced staff take pride in serving the patients and referring physicians the best we can.

We work closely with the UAB Neurofibromatosis clinic led by neurologists and geneticists Dr. Bruce Korf, Chairman of the Department of Genetics at UAB, and Dr. Lane Rutledge, Director of clinical services.

The clinic serves patients and their families dealing with the lifelong medical, psychological and social implications of the various forms of neurofibromatosis and RASopathies.

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